

**Mechanisms of action of plant secondary
metabolites and their effect on
the immune response of parasitised sheep**

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DECLARATION

I hereby declare that the work presented in this thesis is the product of my own efforts and has not been submitted in any previous application for a degree or professional qualification. The work on which it is based is my own except where stated in the text and in the acknowledgements section.

21th April, 2009

Leyla Ríos de Alvarez

The central aim of this study is to investigate the mechanisms of action of plant secondary metabolites (PSM), their direct effect on gastrointestinal nematodes (GIN) and their indirect effect on the local immune response of parasitised sheep. The first approach was through an *in vivo* study using a tannin-rich crop, sainfoin (*Onobrichys viciifolia*), for feeding growing lambs trickle infected with *Trichostrongylus colubriformis*. Thirty-two lambs, 16 weeks of age, were allocated to 1 of 4 treatment groups (n=8) that were offered either grass (G) or sainfoin (S) hay from day -14, while concurrently either infected (+), or not (-) with 12 000 L3 *T. colubriformis* larvae per week, from day 0, for 6 weeks. Liveweight gains were affected by diet ($P=0.002$) and reduced by infection ($P<0.005$). Faecal egg count was reduced in the S+ group compared with the G+ group from days 35 to 42 ($P=0.001$); however, total egg output, worm burdens at day 42 and worm fecundity were similar between diets ($P>0.05$). Feeding sainfoin appeared to enhance immune cell development with tissue eosinophils, mast cells and Pan T cells present in greater concentrations in S+ than in G+ animals. However, further studies are required to determine if the enhanced immune cell development is a consequence of a greater nutrient supply or a direct influence of sainfoin metabolites on local inflammatory responses to the gastrointestinal nematode *T. colubriformis*.

The second approach was to investigate *in vitro* the potential anthelmintic effect of PSM different from tannins, namely plant lectins. Phytohaemagglutinin E3L (PHA-E3L), Wheat germ agglutinin (WGA) and Concanavalin A (Con A or Jack bean lectin) were used with the larval feeding inhibition assay (LFIA), where the direct effect of the lectins at different concentration on the feeding of first stage larvae (L1) was investigated. The results showed that these plant lectins had a profound effect on the feeding of the larvae, especially PHA E3L ($P=0.0006$) at low concentrations, while Con A and WGA were effective at higher concentrations and *T. colubriformis* appeared to be the most susceptible ($P=0.07$) of the three nematode species tested.

In a third approach the intriguing results achieved with the LFIA employing plant lectins were confirmed through an *in vivo* experiment. Twenty-four lambs were allocated to one of four groups (n=6) in a 2x2 factorial design with treatments being either infection (-P: no infection *vs.* +P: mixed infection with *Teladorsagia circumcincta* and *T. colubriformis*) or PHA administration (-L:

no PHA *vs.* +L: oral dose of 80 mg PHA/animal/d), for 6 weeks. Animals received a dose of 167 mg/animal/d of 40.24% w/w semi-purified PHA lectin (equivalent to 80 mg of pure PHA) administered orally five times per week. Compared with their non-dosed counterparts, PHA significantly reduced the faecal egg counts (epg) between days 25 and 36 post infection ($P=0.033$) and had a tendency to reduce the ability of larvae to penetrate abomasal tissue ($P=0.063$), as evaluated by an *in vitro* direct challenge assay (IVDC) used to investigate the effect of lectin treatment on the ability of the larvae to penetrate the mucus layer and migrate to the gastric pits in the abomasal tissue. PHA did not affect cell populations of the intestine. In abomasal tissue of parasitised animals, PHA induced an increase in the number of eosinophils ($P<0.001$) and PAS-positive cells ($P=0.034$). No changes in mucosal mast cells were observed in any of the animals. These results indicate that the oral dosing of PHA may affect local mucosal immune responses during GIN infection, although further studies are required to define both the direct and indirect effects of PHA *in vivo*.

A clear direct effect of lectins *in vitro* on the feeding of first stage larvae was found in the laboratory and clear evidence of an enhanced immune response of the host was shown through both *in vivo* studies. However, there is still more studies to be done to find the mechanisms of action of PSM on the GIN of sheep. The use of PSM or nutraceutical plants, seen from the perspective of being able to influence the immune response of the animals, 'immunoceuticals' is an important target for future studies. If we are able in the future not only to have a direct effect on the worms but also to induce changes in the immune response of the animals through their food, alternative parasite control methods could have an important role to play in commercial farm conditions.

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LIST OF ABBREVIATIONS

Ab-PAS	Alcian blue- Periodic acid Schiff's
ANOVA	Analysis of variance
BSA	Bovine serum albumin
Con A	Concanavalin A
CP	Crude protein
CSL	Sesquiterpene lactones
CT	Condensed tannins
DM	Dry matter
DMI	Dry matter intake
DNA	Deoxyribonucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
EHA	Egg hatch assay
epg	Eggs per g of fresh faeces
FEC	Faecal egg counts
FET	Fetuin
FITC	Fluorescein isothiocyanate
GIN	Gastrointestinal nematodes
GlcNAc	N-acetylglucosamine
GLI	<i>Gliricidia sepium</i>
GNA	Snowdrop lectin
Glu/Man	Glucose/Mannose
<i>H. contortus</i>	<i>Haemonchus contortus</i>
HCl	Hydrochloric acid
HT	Hydrolysable tannin
IC50	Inhibitory concentration 50%
IgG,E, M, A, D	Immunoglobulin G, E, M, A, D
INF-γ	Interferon gamma
IVDC	<i>In vitro</i> direct challenge assay
L1, L2 and L3	Larvae stages: first, second and third (infective stage larvae)
LEU	<i>Leucaena leucocephala</i>
LFIA	Larval feeding inhibition assay
LWG	Liveweight gain
MHC II	Major Histocompatibility complex Class II
MJ	Megajoule
MMC	Mucosal mast cells
MOR	<i>Morus alba</i>
MP	Metabolisable protein
NaCl	Sodium chloride
NAC	<i>Trichanthera gigantean</i>
NaHCO₃	Sodium bicarbonate
Na₂CO₃	Sodium Carbonate
NEE	<i>Azadiractha indica</i>

NKT cells	Natural killer T cells
PF	Paraformaldehyde
PAS	Periodic acid Schiffs
PBS	Phosphated-buffered saline
PEG	Polyethylene glycol
PVP	Polyvinyl pyrrolidone
PVPP	Polyvinyl polypyrrolidone
PHA	Phytohaemagglutinin
PNA	Peanut agglutinin
PSM	Plant secondary metabolite
PVPP	Polyvinyl polypyrrolidone
Q	Quebracho extract
REC	Recovered larvae
REML	Restricted maximum likelihood
SBA	Soybean agglutinin
s.e	Standard error
T cells	"T" stands for thymus
TC cells	Cytotoxic or CTLs
<i>T. circumcincta</i>	<i>Teladorsagia circumcincta</i>
<i>T. colubriformis</i>	<i>Trichostrongylus colubriformis</i>
<i>T. vitrinus</i>	<i>Trichostrongylus vitrinus</i>
TCR	T cell receptor
TCR-CD3	T cell receptor CD3 complex
TEO	Total worm egg output
Th cells	T helper cells, also known as effector T cells or Th cells
TLRs	Toll-like receptors
TOT	Total number of eggs or egg yield
T reg cells	Regulatory T cells
Tris	(Hydroxymethyl) aminomethane
WGA	Wheat germ agglutinin
Zn	Zinc

LITERATURE REVIEW

1.1. INTRODUCTION

Gastrointestinal nematodes (GIN) are a universal problem for farmers and a frequent cause of poor efficiency of livestock worldwide. The control of GIN in ruminants has, in recent years, largely been achieved by the prophylactic and therapeutic use of commercial anthelmintic compounds (Coop and Sykes, 2002). However, the continued use of these products has resulted in the worldwide appearance of nematode resistance (Waller, 1997b). For this reason, and in order to avoid the pollution these chemical compounds contribute to the environment and the food chain, there is much current research into alternative control of gastrointestinal parasitism in ruminants. Some of these proposed alternative methods are long-term approaches including breeding livestock for nematode resistance (Morris *et al.*, 2000) and development of vaccines (Knox *et al.*, 2003; Smith and Zarlenga, 2006). However, the consumption of bioactive forages or plant secondary metabolites (PSM)-rich forages, biological control of parasites through the use of fungi, and nutrient supplementation of the host have also been considered as short term potential solutions (Krecek and Waller, 2006). This current study will focus on the anthelmintic properties of PSM and their importance to alternative GIN control. Novel aspects of this study include the use of lectins in parasite control that could lead us toward a new group of compounds available in crops and seeds from temperate and tropical conditions for alternative parasite control in farms. The mechanisms by

which PSM reduce worm burdens and/or faecal egg outputs will also be investigated.

1.2. ECONOMIC IMPACT OF GASTROINTESTINAL PARASITES

The impact of GIN can have two faces, an impact on the developed world that is different from the impact on the developing countries. In the first case, the effect of GIN in developed countries is essentially related to the costs of treating production limiting nematodoses (morbid effects), whereas in developing countries in the tropics the costs can include mortalities, particularly where the indigenous breeds do not show high levels of genetic resistance to endoparasitic infections (Perry and Randolph, 1999). Anthelmintic treatments used to reduce morbid and mortal effects of worm infection can also have some detrimental effects with regard to ecotoxicity and residues in milk and meat products involved in the use of large amounts of chemical products for controlling GIN in livestock. Besides the secondary consequences, such as pollution, these products can impact on the environment and safety of human food.

Surveys from the late nineteen hundreds showed the high losses in the sheep industry due to parasites, for example in Australia these were estimated in the region of US\$ 222m (McLeod, 1995) and for South America, in a sheep producing country such as Uruguay, losses were estimated at US\$ 42m (Nari *et al.*, 1997). In a more recent study in the UK, Nieuwhof and Bishop (2005) estimated the cost of the three major endemic diseases; these were

GIN, footrot and scab. The estimations included lost performance, preventive measures and treatment of affected animals, resulting as the most costly disease the infestation with GIN, with a cost of over £80m per annum.

The various ways in which diseases and more specifically parasite infections can affect the productivity of animals and herds are shown in Figure 1.1., redrawn from Morris and Marsh (1994), the effects of GIN on the digestion and metabolism and on the ruminant herd are described. Beginning at a simple point of evaluating the effect on the digestion and metabolism, at the end diseases can not only affect single animals but also the entire herd or flock, and even the rate of future genetic improvement. In most cases related with parasitic infections the severity of the losses are in relation to the magnitude of parasite populations. Understandably, successful measurement and assessment of the parasitic population is of high importance (Gruner and Cabaret, 1985).

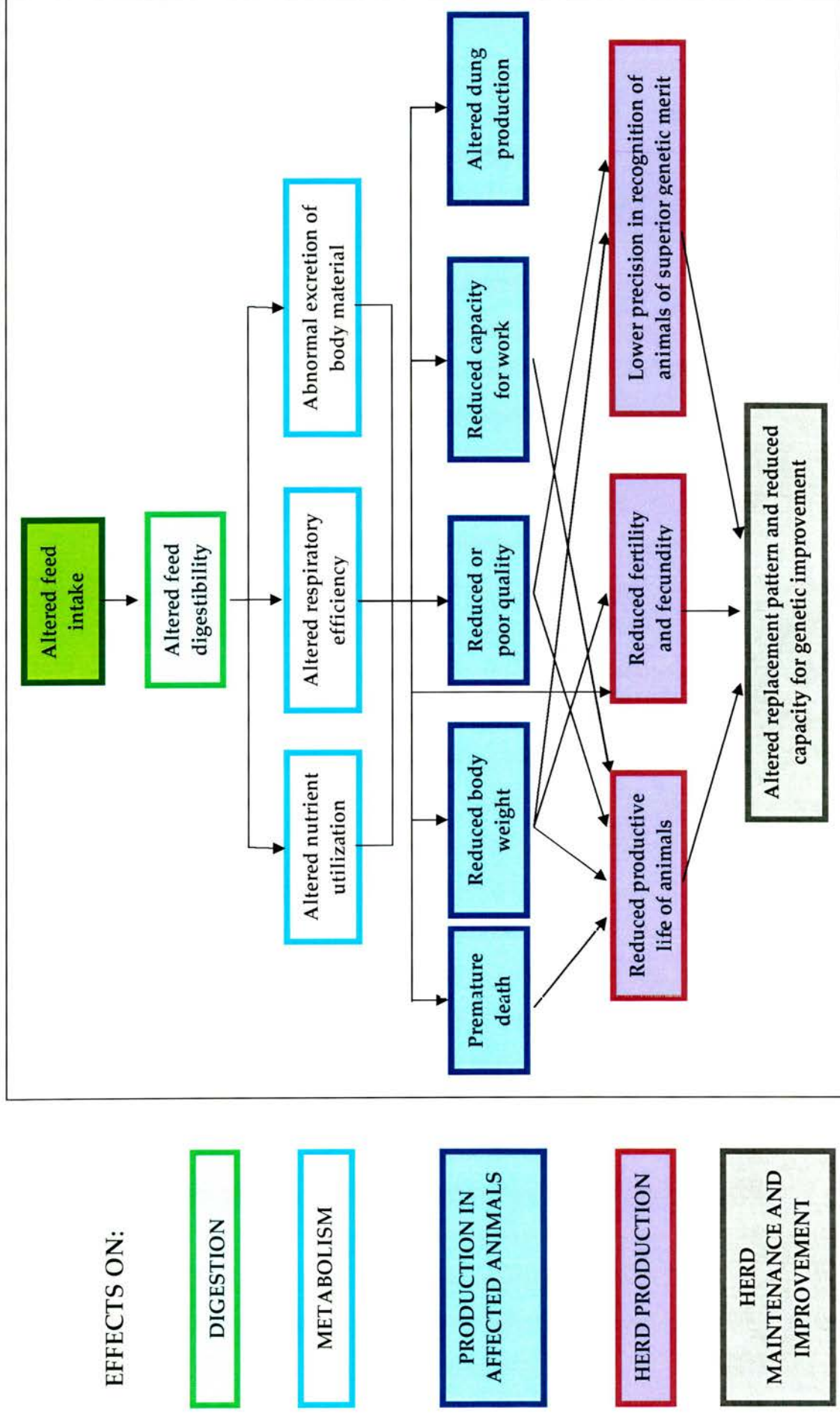


Figure 1.1. Factors related with diseases and parasite infections affecting ruminant animals and herds. Modified from Morris and Marsh (1994).

1.3. COMMON GASTROINTESTINAL PARASITES OF SHEEP

1.3.1. Parasites of the digestive tract

According to the site where they locate there are different parasites in the oesophagus, rumen/reticulum, abomasum and small intestine of sheep (Taylor *et al.*, 2007). In the oesophagus: *Gongylonema pulchrum*; in the rumen/reticulum: *Gongylonema verrucosum*, *Gongylonema monnig*, *Paraphistomum cervi*, *Paraphistomum microbothrium*, *Ceylonocotyle streptocoelium* and *Cotylophoron cotylophorum*; in the abomasum: *Teladorsagia circumcincta*, *Ostertagia leptospicularis*, *Marshallagia marshalli*, *Haemonchus contortus*, *Trichostrongylus axei* and *Parabronema skrjabini*; in the small intestine: *Trichostrongylus colubriformis*, *Trichostrongylus vitrinus*, *Trichostrongylus longispicularis*, *Cooperia curticei*, *Cooperia surnabada*, *Nematodirus battus*, *Nematodirus filicollis*, *Nematodirus spathiger*, *Bunostomum trigonocephalum*, *Gaigeria pachyscalis*, *Strongyloides papillosus*, *Capillaria longipes*, *Moniezia expansa*, *Avitellina centripunctata*, *Stilesia globipunctata*, *Thysaniezia ovilla*, *Thysanosoma actinoides*, *Cymbiforma indica* and *Skrjabinotrema ovis*.

The most common internal parasites of sheep are also classified as: Nematodes or Round worms, Cestodes or Tapeworms and Trematodes or Liver flukes. Nematodes are found in different sites of the host such as the abomasum, small intestine, large intestine and lungs. These studies will focus on the nematode species *H. contortus*, *T. circumcincta* and *T. colubriformis*, the former two are found in the abomasum whereas the latter (*T. colubriformis*) is

located in the small intestine; all are from the superfamily Trichostrongyloidea.

Understanding of the life cycle of these nematodes is important in order to implement effective control programs. All of the economically important GIN are characterized by the adult parasites being located within the digestive tract and their eggs being excreted in the faeces. The non-parasitic part of the cycle occurs on pasture, where the eggs develop into larvae of the first (L1), second (L2) and third stage (L3), which is the infective stage. This life cycle is shown in Figure 1.2. The period of time required for the hatching of the egg and development of the larvae can vary from 5 days to as long as several months, depending on environmental conditions (Whittier *et al.*, 2003).

The life cycle for *T. circumcincta* occurs in 3 weeks. After their ingestion, the L3 exsheath in the rumen and further development takes place in the abomasum. After 18 days and two moults, L5s emerge from the abomasum. Some changes in the cycle happen when ingested L3 stop their development at the stage of early fourth larval stage (EL4), for 6 or more months. This is called hypobiosis or seasonal arrestment.

For *H. contortus* and *T. colubriformis* the life cycle is called typically trichostrongyloid. After the hatch of the eggs into L1 and their development into L3, these are ingested and exsheathed in the rumen and in the abomasum, respectively, the larvae moult twice. In *H. contortus* the larvae develop a lancet shape that is able to pierce the mucosal layer in search of blood. The prepatent period is 2-3 weeks in sheep, which is the time of

development from the moment of infection until the adult is able to lay eggs (Taylor *et al.*, 2007).

For *T. colubriformis* the time for developing eggs to L3 can be 7 to 10 days. After ingestion and exsheathment in the abomasum, the larvae penetrate the mucosa of the small intestine and following two moults L5s can be found under the epithelium around 2 weeks after the infection (prepatent period 2-3 weeks) (Taylor *et al.*, 2007).

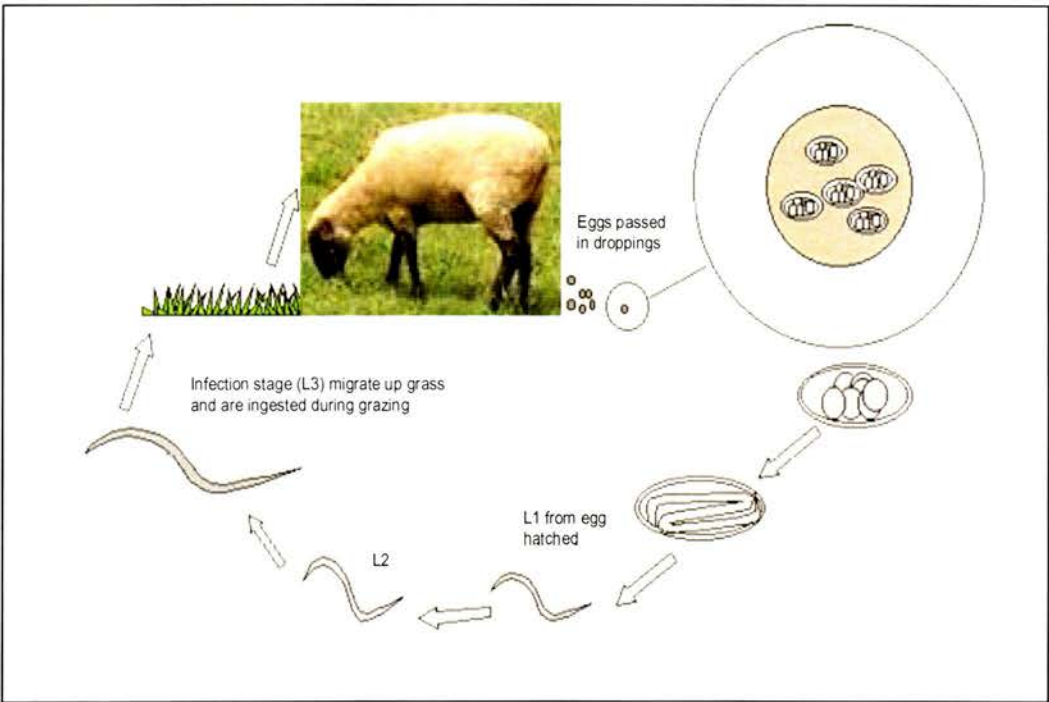


Figure 1.2. Life cycle of gastrointestinal parasites. This cycle includes two phases, one in the sheep with duration of 21 days and a second one on the pasture with duration from 10 to 21 days.

The epidemiology or factors affecting these parasitic nematode infections include temperature and moisture which are critical variables that affect the

survival of eggs and larvae (Sargison, 2008). Round worms require an average daily temperature of 10°C and 50% humidity to hatch, except *H. contortus* which requires more than 15°C. Once the eggs are excreted by the animals in their faeces they hatch to larvae and then factors, such as rainfall, play an important role contributing to the moisture in the faeces that is needed for the hatching of the eggs and development of the larvae. This process of translation involves the active migration of larvae out of the faeces. During this process rain is an important factor contributing to the expositions of the larvae, making it able to migrate onto the herbage and promoting their transportation to the pasture for the continuity of the cycle.

In the tropics temperature is not so variable during the year, and rainfall can be the most important factor influencing the life cycle of parasites. Some studies have demonstrated the close relationship between rainfall and the recovery of infective larvae from pasture (Soulsby, 1982; Agyei, 1997).

Strongyle parasites, specifically *H. contortus* also known as “barber pole” or “wire worm” can have a very damaging effect on sheep and is the parasite that affects most of the livestock in tropical and subtropical areas. However, it is becoming more frequent to find *H. contortus* in some European Countries such as France, Denmark, Sweden and the Netherlands, maybe because of its ability to arrest development at the L4 stage, having a longer period as L4 and a shorter free living stage (O'Connor *et al.*, 2006). Adult *Haemonchus* are obligate blood feeders having the ability to penetrate the mucosa to gain access to the host blood supply. This feeding behaviour causes blood and protein losses and anaemia to the sheep, which can show the characteristic pale mucous membranes especially in the lower eye, and the swelling or

fluid accumulation under the jaw called “bottle jaw” (Schoenian, 2006). Other Strongyle species mainly cause internal tissue damage, reduced appetite and scouring.

Nematodes such as *Teladorsagia* and *Trichostrongylus spp.* are the dominant species from temperate regions because of their survival strategies, including greater resistance to desiccation and better ability to develop in lower temperatures compared with *H. contortus*. In temperate regions, most sheep and lambs are infected in spring and affected during summer and autumn (Sargison, 2008). In milder regions, low temperature is not a restriction for larval development, allowing these species to peak towards late winter and early spring. *Trichostrongylus* can also develop in warmer regions like *H. contortus* (Brunsdon, 1980; O'Connor *et al.*, 2006), Figure 1.3. shows the adequate ranges of temperatures for the development of each parasite species.

1.3.2. Effect of parasites on the host

Clinical signs of parasitism of sheep are also described by Taylor *et al.* (2007):

1. ***T. colubriformis***, (also called “black scour”), can be confused with malnutrition, beginning with reduction of intake, poor growth rates, soft faeces; developing, as the infestation grows, into dark coloured diarrhoea, severe weight lost and even death.

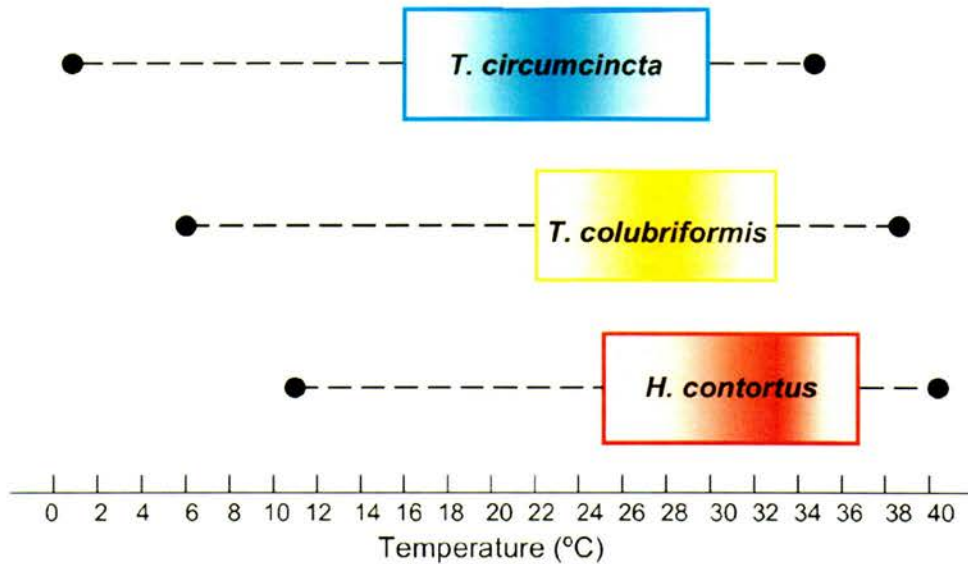


Figure 1.3. Ideal ranges of temperature for the major Strongyle species. Optimum temperatures are indicated by the boxes and the dashed lines cover the entire range for each species. Source: Review by O'Connor *et al.* (2006).

2. With *T. circumcincta*, the signs can be similar to the ones described before for *T. colubriformis*, depression of intake, weight lost and disrupted post-absorptive metabolism of protein. Diarrhoea is not always be present but stained hindquarters are common. After postmortem examination, characteristic lesions can be seen in the abomasum

3. With *H. contortus*, there is an acute anaemia after 2 weeks of infection, because this parasite, present as a medium infection (5000 worms/animal) in a sheep, is able to suck approximately 250 ml of blood each day. The host becomes anaemic and the red cell packed volume begins to drop and in the following days the haematocrit stabilises at a low level, but after days of innappetence and protein and iron losses, the haematocrit falls more, causing

the death of the animal. In *post mortem* examinations of acute haemonchosis from 2000 to 20000 worms can be found in the abomasum and numerous lesions. In cases of hyperacute infestations haemorrhagic gastritis can cause the death of the animal.

1.3.3. Resilience and Resistance

With prolonged exposure to infection sheep may become either “Resistant” and/or “Resilient” to infection. Resistant sheep have the ability to maintain low faecal egg counts (FEC) when challenged with infective larvae and can also regulate parasite establishment and persistence, thus limiting and preventing infection. The more resistant animals are to parasites, the lower pasture contamination will be and this is a characteristic that is moderately heritable. On the other hand, resilience is the ability of animals to maintain their productivity even when they have a parasitic infection. Resilience can be less heritable than resistance; resilient animals can be more productive even with high FEC (Bisset *et al.*, 2001).

In the past before the commercial anthelmintics were used intensively by farmers, animals were able to develop natural resistance to parasites and farmers had the opportunity of keeping as productive animals those more resistant and culling those more sensitive. According to Bisset *et al.* (2001), nowadays with the intensive use of anthelmintics farmers are keeping these weak animals and becoming more and more dependant on chemical products. Figure 1.4. shows when liveweight gain (LWG) is graphed with FEC the animals with higher LWG and lower FEC will be resilient and resistant and therefore the most desirable candidates to stay in the farm. However, other authors assert that selecting animals based on growth and

performance is a way to simultaneously increase the immune status of the animals, because in the partitioning of nutrients, less will be used for immune response (Greer, 2008).

In the following section some basic aspects of immunity and the immune response of the animals towards GIN will be reviewed, since it is these responses which predominantly influence and control worm burdens in the sheep.

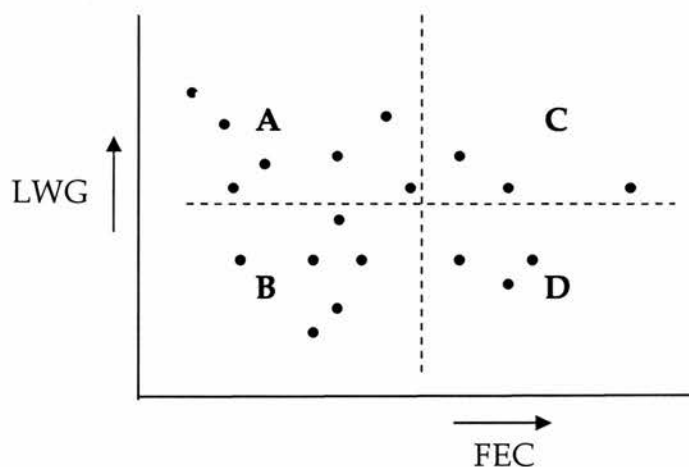


Figure 1.4. Relationship between liveweight gain and faecal egg counts of animals under Strongyle challenge. Animals with low FEC (areas A and B) are defined as “resistant” and those with FEC above average (areas C and D) “susceptible”. Groups under challenge that are able to maintain high LWG (areas A and C) are “resilient” and the group with above average LWG even having high FEC (area C) are “tolerant” animals. Animals performing best are those that show resistance and resilience (area A). Modified and redrawn from (Bisset *et al.*, 2001).

1.4. IMMUNITY OF PARASITISED SHEEP

1.4.1 General aspects related to immunity

The immune system is a complex series of multifactorial mechanisms that provides protection against diseases through the identification of antigens and killing of pathogens. Thus, it is able to identify a large variety of pathogens, such as viruses, bacteria and parasitic worms and differentiate them from the normal cells of the host. This system includes proteins, cells, organs and tissues, which act as a complex network. Overall the immune system can be expressed in two complementary and interacting systems, first the innate immune system and second the adaptive immune system. The innate expression of immunity is found in all animals and its main characteristic is that it is a non-specific defence of the host against other organisms. Innate immunity involves the recognition of specific, or conserved, molecular motifs associated with pathogens. These are recognised through specific pattern recognition receptors such as Toll-like receptors (TLRs) (Perrigoue *et al.*, 2008). This recognition can lead to enhanced macrophage and dendritic cell activity, or inflammatory cell activity involving granulocytes such as mucosal mast cells (MMC) or eosinophils. It is now becoming increasingly evident that there are also links between the innate and acquired immunity, since TLRs on T cells can directly influence their activity (LaRosa *et al.*, 2008). In the second place, the adaptive or specific immune system (also known as the acquired immune system) is a more complex system, developed only in jawed vertebrates, with the ability to recognize and remember (immunological memory) the specific pathogen / antigen and being able to respond in an enhanced and specific way each time

the same pathogen / antigen is presented to the host (Figure 1.5.). This immune system is triggered when pathogens are able to evade the innate immune system and is the basis of vaccination.

The resistance to a helminth infection is a complex process, probably involving both innate and acquired arms of the immune response to be fully effective (Perrigoue *et al.*, 2008).

According to McClure (2000), the development of the acquired immunity to GIN in sheep is affected by the amount of larvae ingested and also varies with the parasite species, for example the immunity induced by *Haemonchus* generally takes longer and it is not as reliable as the immunity developed against *Trichostrongylus*. The steps involved in the development of immunity after the continuous exposure to *T. colubriformis* are:

- 1) From week 5 to 7: rejection of incoming larvae
- 2) Week 10 to 12: depression of worm fecundity, and
- 3) Week 16 to 20: expulsion of adult worms.

Mechanisms involved in the acquired immune response and worm rejection are analyzed in the literature. The main elements of the acquired immune system involve the mechanisms described by McClure (2000):

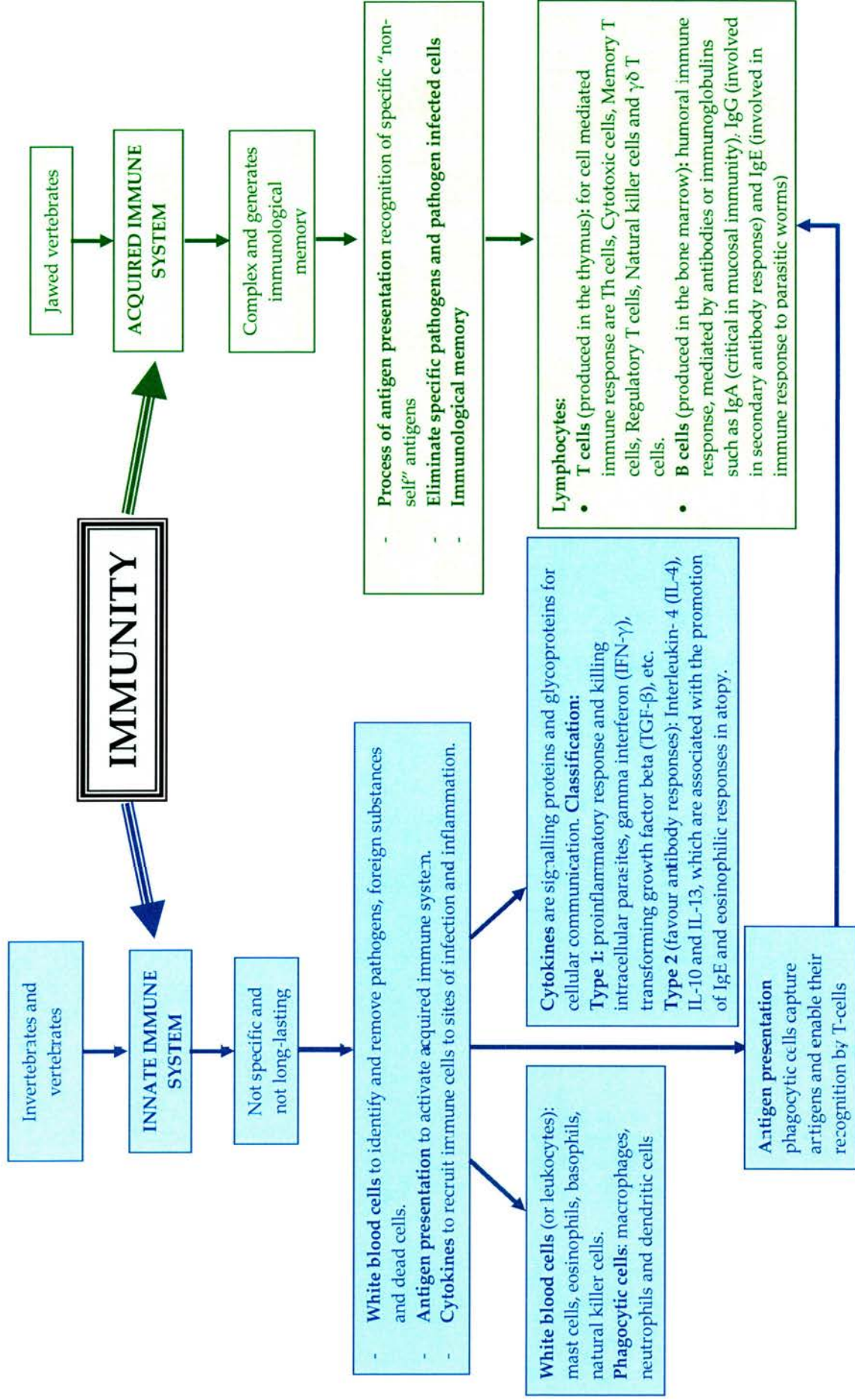


Figure 1.5. The immune response in animals: innate and acquired immune systems, cells, mediators involved and their interactions.

a. Cellular responses: Following infective larval challenge of *T. colubriformis* or *H. contortus* to immune sheep, these larvae are rejected within 24 hours due to a hypersensitivity response (or “rapid rejection”) where MMC are involved with the secretion of a range of potent biological mediators including MMC proteinase (Huntley *et al.*, 1987; Bendixsen *et al.*, 1995). Other cells involved in this process of rejection are the T cells (“T” stands for thymus, the main organ involved in the T cell development), which are a group of white blood cells known as lymphocytes with a special receptor on their surface known as T cell receptor (TCR). Some T cells that have been described are: T helper cells (also known as effector T cells or Th cells), Cytotoxic (TC cells or CTLs), Memory T cells, Regulatory T cells (T reg cells), Natural killer T cells (NKT cells) and $\gamma\delta$ T cells. These latter cells represent a small but important subset of T cells that possess a distinct T cell receptor on their surface, and are particularly abundant in the gut mucosa within the intra-epithelial lymphocyte population and may link innate and adaptive immune responses (Holtmeier and Kabelitz, 2005). Karanu *et al.* (1997) through a study using antigens from the gut of *H. contortus* for the immunization of sheep and goats, found that CD4⁺ T lymphocytes (mature Th cells that express the surface protein CD4) are essential for the development of the immunity induced by gut antigens, and that the antibody worked synergistically with lymphocytes to confer this immunity. According to McClure *et al.* (1995) it appears that CD8⁺ (mature cytotoxic T cells that express T cell receptor to a transmembrane glycoprotein CD8) and WC1⁺/ $\gamma\delta$ ⁺ cells and IFN- γ are also involved in the development of naturally-acquired immunity against *T. colubriformis*, although the precise mechanism remains unclear.

Cytokines: together with chemokines, neuropeptides and neurotransmitters are a complex group of soluble proteins and peptides which function to provide an extensive method of communication between cells and tissues within the organism (Ganea, 2008). Relevant to this study are the cytokines which are produced by Th cells. These cytokines can be divided into two “groups”, namely the Th1-type cytokines which produce for example the pro-inflammatory cytokine Interferon gamma (INF- γ), and Th2-cytokines. Th2 responses predominate in responses to GIN infections, and include interleukins 4 (IL-4), 5 and 13. These cytokine responses are also associated with the production of IgE and allergic hypersensitivity reactions, as well as eosinophilia and mucosal mast cell hyperplasia, which are characteristic features of GIN infections (Balic *et al.*, 2000)

- b. Humoral responses:** humoral immune responses are mediated by antibodies produced and secreted by B lymphocytes.

Systemic: These antibodies are generated in the major lymphoid organs such as the spleen, and are secreted into the blood via the lymphatic ducts. Antibodies are immunoglobulins which are normally employed by the immune system to identify and bind to foreign (non-self) antigens, and are produced by B cells. Immunoglobulins consist of two light polypeptide chains and two heavy chains, joined by disulphide linkages to form a Y shaped structure. The variable regions on both the light and heavy chains determine their antigenic specificity, which recognises and binds to specific regions on an antigen, termed epitopes. This antibody specificity is determined by T cells, which have been primed following

reaction with the antigen peptide MHC II (Major Histocompatibility complex Class II) complex on the macrophage surface. Following the interaction between the T cells and the MHC II-peptide complex, the primed T cells interact with specific B cells (which are recognised through surface epitopes) to stimulate them to clonal division and the production of antibodies. The immunoglobulin isotypes (formally known as classes) are classified according to their heavy chain structure, and consist of IgG, IgM, IgA, IgE and IgD. The main immunoglobulin in blood and most tissues is IgG. In ruminants, there are two IgG sub-isotypes, namely IgG1 and IgG2, although of these IgG1 predominates in ruminant mucosal secretions (Sheldrake and Husband, 1985).

Local: Antibody levels can also influence parasites locally, possibly through their association with mucus, and thus influence parasite rejection (Henderson and Stear, 2006). The isotype IgA is characteristically associated with mucosal, or local, immune responses and is actively transported across the mucosal epithelium. IgA is relatively resistant to proteolysis, so may remain within the gastrointestinal environment without being degraded and able to react against established adult or incoming nematode larvae (Schallig, 2000). However, the precise functions of mucosal IgG1 and IgA in immunity to GIN remains unclear, although IgA may be important in inhibiting nematode larval development, fecundity or motility in sheep (Schallig, 2000; Henderson and Stear, 2006). IgE antibody is often associated with the development of immunity to parasites in ruminants, through the development of mucosal mast cell-mediated type 1 hypersensitivity responses (Miller, 1984). In these responses, IgE is bound to the mucosal mast cell surface via high affinity receptors for the Fc region of the epsilon

heavy chain, and cross linking with antigen (allergen) results in a cascade reaction leading to the release of potent biological mediators from these cells which may directly or indirectly, affect the GINs (Miller, 1984; Rothwell, 1989). Leukotrienes and histamines are examples of inflammatory mediators released from MMC. Leukotrienes are eicosanoid lipid mediators or signalling molecules (there are four families: prostaglandins, prostacyclins, thromboxanes and leukotrienes) naturally produced and are responsible for inflammatory processes. Histamine is derived from the amino acid histidine, which is a neurotransmitter involved in local immune response and physiological functioning of the gut. Both mediators have been found to increase during the process of rejection of *T. colubriformis* (McClure, 2000). These mediators are secreted by MMC and globule leukocytes, and the latter are characteristic of prolonged infection in ruminants.

Neuropeptides: although the central nervous system (CNS) and immune system were always considered separated and independent, in the last two decades they have been shown to have some bidirectional relationships. There are similarities such as the production of soluble mediators: cytokines, chemokines, neuropeptides and neurotransmitters, and also functional links or pathways that connect both systems (Ganea, 2008). Th1-type cytokines produce a proinflammatory response that is involved in killing intracellular parasites and the production of autoimmune responses.

c. **“Non-specific” mechanisms taking part in both innate and acquired immunity:** are local mechanisms involved in parasite rejection, such

as production of mucus, peristalsis, epithelial sloughing and proliferation, fluid and electrolyte movement.

1.4.2 Development of immunity and parasite rejection in sheep

In temperate countries young lambs begin to develop parasitic infection slowly during spring and early summer, because of their low pasture intake or low availability of metabolisable protein (MP) related to their demands for growth, and infection reaches a peak in autumn. During spring and early summer these animals have little innate ability to resist infection and later in autumn they develop a significant immune capability which causes the decrease of their worm burden. After this time when most of the worm burdens have been eliminated, between 10 and 12 months of age, sheep become relatively resistant to serious reinfections. However, this immunity can fail at any age in cases of under nutrition, physiological stress, or concurrent infections or illnesses, and as a result of certain treatments such as corticosteroids. This phenomenon of immune compromise normally occurs in breeding ewes due to pregnancy and lambing stress, and it is known as “postparturient rise”, when immunity is reduced by stress and under nutrition as a consequence of the nutritional demand of lactation (Donaldson *et al.*, 1998). This reduction in immunity results in enhanced larval establishment, development and reproduction, with a peak of FEC 6 to 8 weeks after parturition (Brunsdon, 1970, 1971; Vlassoff *et al.*, 2001). Since much of the increased susceptibility to infection during the peri-parturient period can be ascribed to competition for nutrients

(Coop and Kyriazakis, 1999) the addition of MP can often minimize its impact (Donaldson *et al.*, 1998; Houdijk *et al.*, 2003).

The expulsion of parasitic nematodes from the host has been well documented, including different stages in the case of primary infections: loss phase 1, where a proportion of the infective larvae are lost; plateau phase, the surviving larvae become established and mature and loss phase 2, where almost all the larval population is expelled, leaving a small residual population, the threshold phase. In secondary and further infections, the plateau phase is shorter and a higher rate of worm expulsion occurs in the loss phase (Rothwell, 1989).

Parasite expulsion is also associated with local changes in the mucosa of the alimentary tract of the infected animal, characterised by the proliferation of MMC and eosinophils, goblet cell hyperplasia and the development of intraepithelial globule leukocytes (Rothwell, 1989; Huntley *et al.*, 1995), as has been mentioned above. The immune response of animals varies with species and age. For example, Huntley *et al.* (1995) confirmed that goats were more susceptible to GIN than sheep, and within sheep, ewes were more refractory to infection than lambs. In the sheep activation of MMC as determined by the release of mast cell proteinase, has been shown to coincide with the rejection of worms, suggesting that these cells are involved in the mechanism of worm expulsion (Huntley *et al.*, 1987). Although the development of globule leukocytes is a characteristic feature of GIN infections in ruminants and rodents (MacDonald *et al.*, 1980; Salman and Duncan, 1984), their role in immunity is unclear. Previous work has shown that these globule leukocytes were probably degranulated MMC which migrate from the lamina propria into the gut epithelium (Murray *et al.*, 1968; Huntley *et al.*, 1984), indicating

that these cells have a similar but perhaps enhanced function. Moreover, cells morphologically intermediate between ovine MMC and globule leukocytes can be identified, further suggesting the mucosal mast cell lineage of globule leukocytes in sheep (Huntley *et al.*, 1984).

However, the function of other inflammatory cells is less apparent. Eosinophils may have an important role against larval stages of parasites but not adults (Meeusen and Balic, 2000). Mucin-producing goblet cells have been shown to be important in the rejection of some nematodes in rat and rodent-nematode models (Miller and Nawa, 1979; Miller, 1987; Ishikawa *et al.*, 1993, 1997). Miller (1987) describes mucus as a visco-elastic material that coats the mucosal surface and has three main functions: protection of the mucosa, lubrication for the passage of luminal content and removal of parasites by binding and entrapment. More recently, Nawa *et al.* (1994) found that increased numbers of goblet cells and the secretion of mucus related to parasite infections, suggesting that mucin proteins mediate this response interrupting the adhesion of parasites. While the majority of these studies have been in rodents, studies by Miller *et al.* (1983) have shown in sheep that *Haemonchus* larvae were closely associated with viscous mucus during the phase of immune-mediated expulsion. MMC have been shown to have an important role in the protective and inflammatory response induced following infection with GIN.

There are other ways in which the immunity of the animals against GIN or their resistance/resilience can be enhanced, for example vaccines, genetic improvement of livestock and nutritional supplementation.

Vaccines. Although much work has been performed towards the development of successful vaccines for controlling GIN (Knox *et al.*, 2003; Smith and Zarlenga, 2006; McClure, 2009), up until now there has not been any commercial GIN control through the vaccination of farm animals.

Genetic improvement of livestock. The genetic improvement of livestock in order to make it able to resist parasite infections is the ideal target because this approach could provide a permanent low-cost solution. However, this has not been easy to achieve, because species/breeds that are naturally more resistant to parasites are usually those that are less productive, in terms of milk, meat, wool, etc., and the search for genes that confer resistance to livestock from tropical or temperate regions while maintaining productivity remains in progress (Waller, 1997a, 2006).

Nutritional supplementation. Nutrition has been found to be critical to the development or expression of immunity in the host. It is well known that animals in critical productive stages, such as growth and pregnancy are more likely to be negatively affected by parasites. According to this an extra supplementation not only of energy but also of extra protein in the diet, during these critical phases can be important for the development of a strong immunity in the host against GIN (Houdijk *et al.*, 2003; Torres-Acosta and Hoste, 2008).

1.5. CHEMICAL CONTROL

Traditionally GIN have been controlled effectively using chemical drugs that can be defined, on the basis of their mode of action, as belonging to one of three families; the benzimidazoles, imidazothiazole / tetrahydropyrimidine and macrocycliclactone families. All these drugs were developed in the mid 20th century and smaller doses were required with each new product developed, although the appearance of parasite resistance developed relatively quickly approximately 10 years after each product was released on sale (Waller, 1994; McKellar and Jackson, 2004; Waller, 1997b, 2006). However, ineffective anthelmintic treatment may be due to factors other than resistance; for example incorrect use of the commercial product including the expiry date, accurate dose/animal weight, and incorrect use of the dosing device. If anthelmintics continue to give a poor result, then anthelmintic resistance may have been developed (Torres-Acosta and Hoste, 2008). Nowadays there is no cattle and sheep producing country in the world that does not have a major concern related to anthelmintic resistance (Kaplan, 2004) which has highlighted the need for alternative mechanisms of parasite control.

1.6. ALTERNATIVE CONTROL

Research on natural alternatives for the control of GIN of ruminants has become more important, and there is an urgent need for new, safe and effective compounds with no resistance from the worms, with minimal concerns related to toxic residues in human food or potential pollution to the

environment (Hoste *et al.*, 2008b). One such alternative is the use of plants and plant products which contain anthelmintic properties. However, an important problem of these alternatives methods of control is their partial efficacy or variability of results depending on worm species, and differences in the concentrations of the active compounds responsible for the anthelmintic effect (Jackson and Miller, 2006). For example, the concentration of active compound may vary with the season and stage of plant development at harvest, and the post-harvesting treatment. Most of these known active components are within a group collectively termed as PSM, which have been studied in depth in the last few years, both *in vivo* and *in vitro*. However, there are still issues related to the use of PSM that have not been answered such as: mode of action against worms, sources that cause of variability in their efficacy, exact biochemical characterisation of the compounds and optimal conditions for their use or application (Torres-Acosta and Hoste, 2008). Moreover, it is generally accepted that the PSM so far studied have been less effective than the traditional anthelmintics employed to date.

1.6.1. Plant secondary metabolites

PSM have little or no nutritional value and no known function in the primary metabolism of plants (Greathead, 2003; Iason, 2005). However, they may have other functions related to the plant, such as protection against pathogens and herbivores, a role to play in inter-plant competition and abiotic stresses, beneficial organism attractors (pollinators), and can also be involved in the defensive mechanisms in response to a stressor, e.g. repeated cutting (Greathead, 2003). They can be found in the foliage, seeds or in the

whole plant used in practical feeding. PSM include a long and diverse list of compounds such as tannins, lectins, saponins and alkaloids. In the next sections, the two predominant PSM of interest in these studies, namely tannins and lectins, will be characterised including their properties and their use in parasite control of ruminants.

1.6.1.1. Tannins

The most widely studied PSM are tannins, because they are widespread in plants and seeds and also because of their high biological activity. These compounds are divided into two groups: hydrolysable tannins and proanthocyanidins (or condensed tannins-CT) (D'Mello, 2000; Waghorn and McNabb, 2003). Hydrolysable tannins are polyesters of gallic acid and simple sugars, while CT are polymers of flavonoids (Khanbabaei and van Ree, 2001).

Tannins are well known for their medicinal properties, including anticancer effects (Yi *et al.*, 2006), antifungal activity (Ngono Ngane *et al.*, 2006) and of main importance for this study, anthelmintic effect. Some important research using tannins and tannin-rich plants for this purpose will be described in this work.

1.6.1.2. Lectins

Originally, the term lectin was restricted to soluble, multivalent proteins capable of agglutination and limited to proteins of plant origin. However, today the term is used for all types of carbohydrate-binding proteins that do not catalyze reactions with their ligands or do not alter them

enzymatically (Goldstein *et al.*, 1980; Varki *et al.*, 1999). Lectins have been found in plants, bacteria, slime moulds, sponges, other invertebrates and vertebrates. Many lectins have been detected, isolated and characterised from a wide range of sources and their biological activities have been extensively studied (Rüdiger, 1984). Their most striking property is their ability to stimulate lymphocyte proliferation, in particular T cells, which can result in enhanced immune responses including the production of antibodies (Rüdiger, 1984).

Lectins were discovered by Peter Stillmark at the end of the 19th Century in an Estonian University. He found that some proteins extracted from castor beans (*Ricinus comunis*) were able to agglutinate erythrocytes. Years later in 1960, Peter Nowell showed that some lectins, such as kidney bean (*Phaseolus vulgaris*) agglutinin or phytohaemagglutinin (PHA) were able to induce mitotic activity in lymphocytes (Kilpatrick, 1999). The mechanisms of action of the PHA for producing mitogenic effect were not clear. Nowell (1960) stated that "perhaps the action of the lectin on leukocytes involved the cell surface, possibly altering the cell membrane to allow the entrance of substances (essential factors such as serum components or cellular products) which had the mitotic effect". He also concluded that the lectin did not induce the mitosis *per se*, but had an effect of transformation on monocytes and large lymphocytes to a state capable of division. At that time, lymphocyte function was uncertain, T and B-cells, lymphokines, and T-cell receptors were still not discovered, and the role of the MHC was still an enigma (Kilpatrick, 1999). Research performed by Nowell (1960) using PHA led to the discovery of other lectins and their inhibitors or specific sugars that were able to reversibly stop the mitogenic action of the lectins. While lectins, such as PHA or Concanavalin A (Con A), are mitogenic for lymphocytes,

many other lectins, such as wheat germ agglutinin (WGA) and tomato lectin, have no effect on mitosis and a few lectins have now been shown to be anti-mitotic (Greene and Waldmann, 1980; Nachbar *et al.*, 1980; Kilpatrick, 1999). From comparative studies it was shown that mitogenic lectins bound to the T-cell receptor complex whereas the antimitogenic lectins did not. Kilpatrick (1999) summarized the main events involved in the mitogenic stimulation of lymphocytes:

1. Binding of the mitogen to the lymphocyte cell surface
2. Altered cell permeability
3. Phosphorylation of the protein
4. Synthesis of IL-2 receptors
5. Synthesis of lymphokines
6. Synthesis of DNA
7. Mitogenic process

Morgan *et al.* (1976) produced another striking discovery with the demonstration of the production of a T-cell growth factor (now known as IL-2) in conditioned medium from normal human lymphocytes stimulated by PHA (Sharon and Lis, 2004).

Lectins have potent immunostimulatory properties and they have been used widely as non-specific mitogens in artificial systems for T cell activation. PHA and Con A bind strongly to many cell membrane glycoproteins, including the T cell receptor CD3 (TCR-CD3) complex, inducing an activation response and proliferation in most of the T cells (Kay, 1991). Plant lectins also have been described as being able to induce pro- or anti-inflammatory actions activating or inhibiting neutrophil migration (Alencar *et al.*, 2003, 2005). In addition to their effects on immune cells,

studies in rats have shown that red kidney bean lectin, PHA, can stimulate the production of stem cells in intestinal crypts and promote rapid growth of the gut (Oliveira *et al.*, 1988; Grant, 1999). In addition, administration of PHA resulted in an accelerated growth and precocious maturation of the gastrointestinal tract of rat pups (Bardocz *et al.*, 1989; Banwell *et al.*, 1993; Kruszevska *et al.*, 2003; Linderroth *et al.*, 2005, 2006b). Linderroth *et al.* (2006a) also studied the effects of enteral *vs.* parenteral administered lectin, finding, that enteral exposure to PHA caused binding of PHA to the epithelium of the small intestine, gastrointestinal growth and reduced intestinal absorption, in this case, it had no effect on the growth of the gastrointestinal tract but led to an increased weight of extra-intestinal organs, such as liver and spleen.

Some lectins have recently been used to study the roles of carbohydrate structures in host-parasite interactions. Ohashi *et al.* (2007) studied host specificity of *Neobenedenia girellae*, which is a monogenean skin parasite of marine fish that causes high economic losses. They identified a glycoprotein Wap 65-2 that was able to attach to the oncomiracidia of the parasite. A panel of lectins has been used to study host- *Schistosoma bovis* relationships and identify possible glycans and glycoproteins involved in the interactions (Ramajo-Hernández *et al.*, 2007).

Recent studies have indicated that lectins are also centrally involved in many aspects of vertebrate and invertebrate immunity and biology. For example, galectins are a family of animal lectins that play an important role in the immune system of animals and also are targets for development of new anti-inflammatory and anti-cancer treatments (Leffler, 2008). Galectins (formally termed S-type lectins), are also a key component of the innate immunity

system and important in pathogen recognition in marine invertebrates (Kim *et al.*, 2008).

To summarise, these latter results suggest that the effects of lectins are wide ranging, acting directly or indirectly on numerous targets and influencing the dynamic responses of both the acquired and innate arms of the immune response. Moreover, the lectin binding can be used to identify factors important in host-parasite interactions.

1.6.2. Anthelmintic effect of tannins and tannin-rich plants.

Results *in vitro* and *in vivo*

1.6.2.1 *In vitro* effect of tannins and tannin-rich plants

The antiparasitic effects of PSM and CT in particular, have been demonstrated *in vitro* using various plant extracts. One commercial plant extract widely used for these experiments is Quebracho (Q), which contains around 70-76% CT. This extract is produced from a subtropical tree *Schinopsis lorentzii*, of the family Anacardiaceae. The incubation of *T. circumcincta*, *Trichostrongylus vitrinus* and *H. contortus* larvae, in different concentrations of Q extract, was found to have no effect on larval development to the third infective stage. However, the higher the concentration the greater the effect on the viability of the larvae, showing a linear relation between larval viability and Q concentration (Athanasiadou *et al.*, 2001) (Table 1.1.).

Table 1.1. Some *in vitro* studies performed using Quebracho-Q and plant extracts (mixture of plant secondary metabolites-PSM) for gastrointestinal parasite control.

Forage species	Parasite species / Assays	Results	Authors
<i>Schinopsis lorentzii</i> (Quebracho)	<i>T. circumcincta</i> , <i>T. vitrinus</i> and <i>H. contortus</i> Larval development / viability assay	No effect of Q on the different species of larvae, more than 98% of all the larvae developed to L3 infective stage. Linear relation between larval viability and Q concentration	Athanasiadou <i>et al.</i> (2001)
<i>Lotus corniculatus</i> , <i>Lotus pedunculatus</i> , <i>Hedysarum coronarium</i> , <i>Onobrychis viciifolia</i> , <i>Dorycnium rectum</i> , <i>Dorycnium pentophyllum</i> , <i>Rumex obtusifolius</i>	<i>T. colubriformis</i> Egg-Larval development assay	Greater effect of plant extracts on larval development when added before hatching the eggs, than after hatching the eggs	Molan <i>et al.</i> (2002)
<i>L. corniculatus</i> , <i>L. pedunculatus</i> , <i>H. coronarium</i> , <i>D. rectum</i> , <i>D. pentophyllum</i> ,	<i>T. colubriformis</i> Egg hatch and larval development using eggs extracted from faeces of animals fed with different forages	Host diet affects subsequent larval development. <i>D. rectum</i> and <i>D. pentophyllum</i> had higher ovicidal and larval development inhibitory effect	Niezen <i>et al.</i> (2002)
<i>Chicorium intybus</i> (CT and crude sesquiterpene lactones-CSL)	<i>Dictyocaulus viviparus</i> and GIN (mainly <i>T. circumcincta</i>) Larval migration inhibition assay	CT was more effective than CSL inactivating L1 and L3 lungworm and L3 gastrointestinal larvae in rumen fluid. CSL was effective against L3 lungworm larvae in abomasal fluid	Molan <i>et al.</i> (2003)

Other plant extracts (mixture of PSM) have also been investigated for their anthelmintic properties *in vitro*. Molan *et al.* (2002) and Niezen *et al.* (2002) tested the effects of CT extracted from different temperate forages on the

viability of eggs and L1 larvae of the sheep intestinal nematode *T. colubriformis*. In the first study, Molan *et al.* (2002) found that the extracts were significantly more effective in inhibiting larval development when they were added before the eggs hatched, than when they were added after hatching.

Additionally, Niezen *et al.* (2002) evaluated the effect of these CT extracts on larval development *in vitro* and on pasture. They found that the effects *in vitro* were not an accurate estimation of what was happening in field conditions. The laboratory assays performed used eggs extracted from the faeces and contrary to this, in field conditions the eggs remained affected by the CT for many days, which had a marked effect on the subsequent development of *T. colubriformis* larvae. They concluded that if CT were able to break the life cycle of nematodes and thus reduce the contamination of pastures with viable eggs, this could be a useful alternative to control parasitism in grazing systems.

When testing CT from forage extracts, Molan *et al.* (2003) also isolated sesquiterpene lactones (CSL) from chicory (*Chicorium intybus*) and tested it on the motility of the first and third stage larvae of deer lungworm (*Dictyocaulus viviparus*) and GIN *in vitro*. In this study they found that CT were more effective than CSL in inactivating L1 and L3 lungworm and L3 gastrointestinal larvae in rumen fluid, but CSL were more effective against L3 lungworm larvae in abomasal fluid. Although all these *in vitro* results are interesting, it is difficult to compare them with *in vivo* conditions; for example, sometimes the concentrations used *in vitro* are not biologically possible to achieve with farm animals, making results difficult to apply to

real farm conditions. In addition, there are pH and physico-chemical differences in the *in vivo* experiments that cannot be replicated in laboratory conditions and could affect results achieved.

1.6.2.2. *In vivo* effect of tannins and tannin-rich plants

Results using different PSM-rich forages for controlling GIN in small ruminants are contradictory because of the differences in parasites species, their habitat (abomasum *vs.* intestine) (Athanasiadou *et al.*, 2001; Athanasiadou and Kyriazakis, 2004) and the host species (sheep *vs.* goats) (Hoste *et al.*, 2008a) tested in various experimental studies. Additional differences in the age of animals infected (adults *vs.* young animals) (Waghorn, 2008), their previous experience with parasites (adults with previous experience *vs.* young naïve animals) (Hoste *et al.*, 2008a) and differences in length of time on PSM forages (short-term studies *vs.* long-term studies) (Athanasiadou and Kyriazakis, 2004), can also make these results difficult to interpret. In addition, most of the PSM-rich forages used are selected because of their high tannin content, not taking into account that these plants also contain other PSM such as lectins, saponins, etc. All these factors make it difficult to confirm that there is clear evidence of a positive anthelmintic effect.

However, relevant studies *in vivo* are summarised in a recent review (Hoste *et al.*, 2006), using different tanniferous forages with sheep, goats and deer. Important details, such as level of CT in the forages, effects on the performance and resilience of the animals and the main effects on nematode populations, are shown. In most of the studies described, the performance of

the animals is improved through the intake of these bioactive forages and also these forages have been associated with antiparasitic effect, generally expressed as FEC reduction and effect on female worm fecundity. This review also reinforces the idea that results achieved with tanniferous plants can vary according to plant species/stage, the environment in which the plant is grown, the stage and composition of nematode populations and possibly also the host species. Some of these *in vivo* studies are described in detail below.

Niezen *et al.* (1998) looked at the effect of grazing on different CT forages on faecal egg count and performance of naturally infected ewe lambs. They found that animals grazing *Hedysarium coronarium* had a reduction in the numbers of intestinal *Trichostrongylus* and in parasite density in the abomasum and intestine (nematodes/g organ); also animals grazing *L. pedunculatus* maintained a high level of performance despite high FEC and worm burdens.

Paolini *et al.* (2003c, 2004b), Athanasiadou *et al.* (2005a, 2005b) and Tzamaloukas *et al.* (2005) performed *in vivo* experiments using artificially infected animals. In the first experiment, Paolini *et al.* (2003c) found different effect of Q on adult worm populations *vs.* infective incoming larvae. In the second study, Paolini *et al.* (2004b) found no effect of either the Q extract or the sainfoin hay on the abomasal species *H. contortus* in young goats. Athanasiadou *et al.* (2005a, 2005b) artificially infected sheep with the intestinal parasite species *T. colubriformis* using both confined and grazing animals; they found sainfoin had an effect on the total egg output in confined lambs. By contrast, in the second experiment (Athanasiadou *et al.*, 2005b) the PSM-rich forages tested, including sainfoin showed no anthelmintic effect.

On the other hand, Tzamaloukas *et al.* (2005) studied the effect of short-term grazing of different PSM-rich forages on the viability and fecundity of an established adult population and the establishment and development of incoming infective larvae of *T. circumcincta*, using naïve animals infected with this parasite. Their results suggest that the best effect was achieved with chicory, with the reduction of the established adult worm population; the effect achieved with the other forages was minimal.

All these are clear examples of the variability among studies and results achieved using *in vivo* conditions. To summarize, PSM-rich forages had an effect against intestinal but not abomasal nematodes, in sheep and goat, although their action was not consistent across the grazing seasons. In both indoor and foraging conditions the quality of feed (protein content, PSM content) can change from one experiment to another and also from one season to other, influencing the results achieved. The inconsistencies observed between parasite species, habitat (abomasal or intestinal), might be due to the formation of complexes between the tannins and the proteins. It is accepted that the interaction between protein and CT is pH dependent, CT bind with protein in near neutral pH (pH 3.5-7.5), as occurs in the rumen, forming CT-protein complexes (Jones and Mangan, 1977). The formation of these complexes may result in the inability of tannins to exert an effect on the abomasal parasites, but once tannins are in the intestine, differences in pH and the presence of surfactants could cause disassociation, making it possible for the free tannins to negatively affect the parasites (Martin *et al.*, 1985).

Recently, other compounds such as cysteine proteinases have been studied because of their direct effect on the nematodes. Stepek *et al.* (2005, 2006) studied the effect that plant cysteine proteinases had against *Heligmosomoides*

polygyrus and *Trichuris muris*, two rodent GIN with different habitats, the anterior part of the small intestine and large intestine, respectively. They found that these compounds caused marked damage to the worms, including digestion and removal of their cuticles, also affecting also their motility and reducing the faecal egg counts of mice infected with *Trichuris muris*.

The next PSM to be described are lectins which have been less studied than tannins for anthelmintic properties but will be the central focus of our studies.

1.6.3. Lectins and gastrointestinal parasite infections

The direct or indirect effect of plant lectins, present in seeds or forages, on the GIN population of ruminants has not been previously studied. However, the intriguing finding of enhanced intestinal mucosal cell proliferation following the oral dosing of kidney bean lectin to rats (Grant, 1999) and more robust gut responses to trauma (Grant *et al.*, 2008), would suggest that lectins may be able to affect host-parasite interactions. It may be possible to enhance the host's natural immune or innate mechanisms of resistance through the intake of lectins, and thus assist in the control of GIN infections. However, it is also known that some lectins such as ricin are highly toxic. Vasconcelos and Oliveira (2004) summarised in a recent review that high intakes of some plant lectins can adversely affect gut metabolism, leading to damage to luminal membranes, loss of gut epithelial cells, crypt/villus disruption and interference with nutrient digestion and

absorption. As a result, the growth and health of consuming animals may be impaired (Grant, 1999). Therefore, if such an approach is employed, very careful consideration should be undertaken as to the dosage and known toxicity of the lectin. In humans, it has been suggested that lectins may also be involved in the incidence of autoimmune diseases such as rheumatoid arthritis (Cordain *et al.*, 2000). This is a complex disease characterized by persistent inflammation of the synovium, local destruction of bone and cartilage and a variety of systemic manifestations which may ultimately result in functional disability. Autoimmune diseases or conditions occur when the body is unable to distinguish self from non-self antigens, resulting in the generation of antibodies which react with the hosts own antigens. Consumption of cereal grains and legume lectins, leading to a chronic condition through repeated antigenic stimulation has been suggested as a possible causative mechanism. However, this has only been shown *in vivo* when lectin was injected directly into joints (Cordain *et al.*, 2000)

PHA is a strong mitogenic and haemagglutinating lectin and comprises of five different isolectin forms (E for erythroagglutinating; L for lymphoagglutinating): E4, E3L, E2L2, EL3 and L4 (Leavitt *et al.*, 1977). It has a particularly high resistance to proteolytic degradation *in vivo* (Herzig *et al.*, 1997). It can survive in the intestinal lumen in an intact and fully functional form and interact with epithelial cells in the gut and modify their metabolism and has serious adverse effects on animals if consumed in high amounts (Grant, 1999). However, low or moderate intakes modified intestine metabolism in rats without triggering adverse effects (Grant, 1999; Grant *et al.*, 2008). Thus, employing appropriate intakes of lectin in the diet of sheep, it may be possible to maximise the effectiveness of the lectin as an alternative

or additional control strategy for parasites while minimising any harmful side effects.

Studies relating to the binding and degradation of lectins by components of the rumen liquor and the fate of lectins in the ovine gut show that many lectins bind to feed components and bacterial cells, and are able to resist breakdown in the rumen (Baintner *et al.*, 1993a, 1993b). An important feature of some plant lectins is their remarkably high resistance to proteolysis and stability over a large range of pH, and studies have shown that PHA, Con A, the snowdrop lectin (GNA) and WGA are able to traverse the rat and human small intestine without significant degradation (Vasconcelos and Oliveira, 2004). This strengthens the evidence that at least some lectins have the potential to be employed to control nematode parasites in the abomasum or small intestine, because of their relative stability and retention of biological activity during passage in the gastrointestinal tract.

Lectins may also interfere directly with metabolism of GIN themselves. Tobata-Kudo *et al.* (2005) showed that some lectins suppressed migration of *Strongyloides ratti* L3 *in vitro*. It was suggested that the chemosensory function of the parasite was disrupted, using Con A, WGA and soybean agglutinin (SBA) at a concentration of 0.5 mg/ml. The lectin-binding sites of the parasite were also studied using fluorescein isothiocyanate (FITC)-conjugated Con A, and were able to show that the lectin was able to bind strongly to the area of the amphids, the buccal cavity and with less intensity to the surface of the cuticle.

In research on plant lectins and *Schistosoma mansoni* infection (schistosomiasis is a tropical human parasitic disease) performed by Reis *et*

al. (2008), described that the immune response to schistosomiasis is Th1-like at the initial stage of infection, changing after to a Th2-like response after oviposition. They were studying the immunomodulatory effect of lectins, and found no effect on changing the Th2 response of the infection.

Binding studies on filarial parasites such as *Wuchereria bancrofti* and *Brugia patei*, have been done using plant lectins (Rao *et al.*, 1987, 1989). The authors were able to find differences between these parasites according to their surface-lectin binding properties.

Another study showed that plant lectins can play an important role in the parasite-host interaction in plants. This refers to the cyst nematode *Heterodera avenae* that invades the roots of a host cereal plant, increasing the levels of barley lectin and WGA in the roots. It is not known whether this increase is due to a specific reaction of the plant to the nematodes or due to the stress caused by the nematode invasion (Oka *et al.*, 1997).

Finally, plant lectins have also been studied as a way of controlling plant nematodes, such as the pinewood nematode (Gaofu *et al.*, 2008). In this study two lectins were purified from corms of *Pinellia ternata* and *Lycoris radiata* (*P. ternata* agglutinin-PTA and *L. radiata* agglutinin-LRA) and their effect on the pinewood nematode was measured, demonstrating that these lectins had a strong anti-nematode effect *in vitro*.

The above section has described briefly and in general terms, the immune responses which are involved in controlling GIN infections and the factors involved which can influence the host-parasite interaction. Although the link between the intake of PSM and these immune reactions may not be

immediately apparent, it is clear that any process which can enhance the qualitative or quantitative aspects of this immunity is likely to be of benefit to the host. The link between immunity related cells and the intake of PSM for parasite control is unclear. An approach that can help to clarify this link will be included in this study. However, lectins have not been investigated for parasite control, this group of compounds have been employed widely by immunologists as stimulating proteins, for example both PHA and Con A, which are routinely employed to stimulate T cells in lymphoproliferation assays (Kay, 1991). In this study, the effect of lectins, both directly on the nematodes, and indirectly by their effect on the immune response of the host, will be investigated.

1.7. AIMS AND OBJECTIVES OF THIS THESIS

The aims of this PhD study are:

1. To study the effects of feeding sainfoin to experimentally *T. colubriformis* infected sheep and investigate the anthelmintic effect of this plant and its effect on the local immune response of the host.
2. To study the direct effects of plant lectins on nematode larvae *in vitro* by employing the larval feeding inhibition assay.
3. To investigate the anthelmintic effect of tropical plant extracts on GIN, and define the involvement of lectins and mode of action.
4. To investigate the potential of a lectin extracted, in a semi-purified form, from *Phaseolus vulgaris* seeds (Phytohaemagglutinin-PHA), as a method of alternative control of GIN *in vivo*, and investigate its mode of action including effects on the local immune response.

EFFECT OF SAINFOIN (*Onobrychis viciifolia*) ON LOCAL CELLULAR RESPONSES TO *Trichostrongylus colubriformis* IN SHEEP

2.1. INTRODUCTION

The prospect of exploiting the potential anti-parasitic effects of plants that contain PSM in order to provide an alternative to chemical prophylactic treatments in grazing livestock has stimulated research in this area (Ramírez-Restrepo and Barry, 2005; Hoste *et al.*, 2006; Waghorn, 2008). While the identification of plants with antiparasitic properties has been possible, their incorporation into grazing systems has frequently been restricted due to agronomical issues. Sainfoin (*Onobrychis viciifolia*) is a nutritious leguminous plant that has been used in Europe for more than 300 years as a source of high quality, dust free hay for feeding heavy working horses (Frame *et al.*, 1998). Additionally, its bloat free characteristics, makes it an excellent candidate for fattening lambs (Koivisto and Lane, 2001).

Sainfoin was found to contain a variety of PSM, such as phenolic glucoside compounds, flavonols, flavonols glycosides, lectins and a mixture of procyanidin and prodelphinidin type CT (Kouchalakos *et al.*, 1984; Marais *et al.*, 2000), and for this reason has been tested for anthelmintic activity *in vitro* (Paolini *et al.*, 2004a; Barrau *et al.*, 2005) and *in vivo* (Paolini *et al.*, 2003b, 2005a, 2005b). Furthermore, sainfoin's ability to withstand grazing (Koivisto and Lane, 2001) makes it an ideal candidate to provide a therapeutic antiparasitic effect in infected livestock.

In general, positive antiparasitic effects have been achieved on intestinal worms where sainfoin consumption resulted in lower faecal egg counts and total egg output (Athanasiadou *et al.*, 2005a; Paolini *et al.*, 2003a, 2003b, 2005b). Although Paolini *et al.* (2005a) found no effect of the intake of sainfoin on the abomasal parasite *H. contortus* in goats, Heckendorn *et al.* (2006, 2007) showed an effect of sainfoin on the same parasite in sheep, when animals were offered sainfoin post-infection.

Despite these previous studies on the anti-parasitic activity of sainfoin, the mechanisms of its action are unclear. Both PSM and, in particular, CT, which are the active compounds in sainfoin (Hoste *et al.*, 2005; Paolini *et al.*, 2005b) may act directly and/or indirectly on the parasites by enhancing host immune response. Therefore, the purpose of the present study was to further investigate the effect of sainfoin consumption on nematodes, and in particular to define its mode of action in sheep trickle infected with *T. colubriformis*. During the first few weeks of infection, the direct effect of the intake of sainfoin on the parasitism of the animals was measured through the reduction of the faecal egg counts and total egg output. Indirect effects, including enhanced responses of the local inflammatory cell populations may be evident at the terminal time point, since these local inflammatory cellular reactions are known to play a pivotal role in immunity to GIN infections in rodents and ruminants, but may require prolonged worm stimulation for the full expression of some components such as globule leukocytes (Miller, 1984).

2.2. MATERIALS AND METHODS

2.2.1. Animals, housing and feeds

Thirty-two weaned 16 week-old Texel x Scottish Greyface lambs previously reared indoors under conditions that excluded exposure to nematodes, were allocated to 1 of 4 groups (n=8) that were balanced for sex (castrated males and females) and initial liveweight (34.5 ± 0.124 kg). Within the animal house, animals were randomly allocated in individual pens. From day -14, two of the groups were offered grass hay (diet G) while the remaining two groups were offered sainfoin hay (diet S), the chemical composition of each diet is given in Table 2.1. All feeding was *ad libitum* and animals were also given free access to fresh water.

Table 2.1. Chemical composition of a pooled sample of the grass and sainfoin hays offered to animals that were infected, or not, with *T. colubriformis*. (% phenolics and tannin concentrations are expressed on a dry matter basis).

	Grass Hay	Sainfoin Hay
Dry Matter (g/kg fresh matter)	836	886
Crude Protein (g/kg DM)	83	93
ME (MJ/kgDM)	8.8	8.4
FME (MJ/kg DM)	8.6	8.1
DOM (D-value, % DM)	54.9	52.6
Ash (g/kg DM)	70	51
Phosphorus (g/kg DM)	2	1.4
Total Phenolics (Catechin equivalents)	8	18
Total Tannins (Quebracho equivalents)	< 20	20

ME = Metabolisable energy, FME = Fermentable metabolisable energy estimated by in vitro gas production, DOM = Digestible Organic Matter.

2.2.2. Experimental design

Within each feeding regime, 1 group (infection +) received a trickle infection with 12000 *T. colubriformis* L3 infective larvae per week from day 0 until day 42 that was administered in three doses of 4000 larvae per dose on alternate days. The remaining group within each dietary regime was maintained as a non-infected control (infection-). This resulted in a 2x2 factorial design with the groups being G+, G-, S+ and S- (Figure 2.1.).

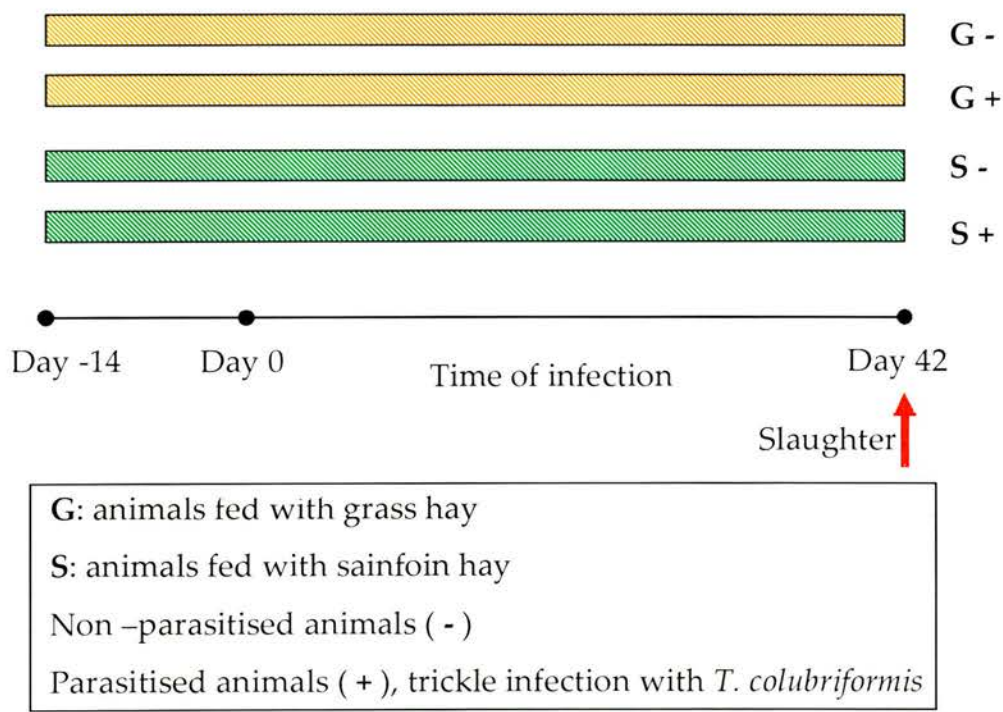


Figure 2.1. *In vivo* experiment design. G and S represent the different feed arrangements and '+' and '-' the parasite treatment, being '-' the control.

2.2.3. Measurements and sampling procedures

2.2.3.1. Food composition

Pooled weekly samples of the hays offered and refused were taken and analysed for determination of crude protein (Kjeldahl method), total phenolics (Price and Butler, 1977) and extractable CT content (Porter *et al.*, 1986). Estimation of the energetic feed value and digestibility of the feeds was obtained through *in vitro* gas production using rumen fluid (Menke and Steingass, 1988). The grass and sainfoin hays contained 8.3 and 9.3 g of crude protein and provided 8.8 and 8.4 MJ of metabolisable energy per kg of DM, respectively (Table 2.1.).

2.2.3.2. Food intake and liveweight

Fresh hay intake was measured daily with subsamples of feed offered and refused dried to allow estimates of daily dry matter (DM) intake. Lambs were weighed weekly, during the morning of the same day and also on the final day of the experiment prior to slaughter, for the estimation of liveweight gain.

2.2.3.3. Plasma analysis

Blood samples were collected weekly by jugular venepuncture into heparinised vacutainers (Becton Dickinson, UK). Blood tubes were centrifuged at 2500 rpm for 15 min and the plasma was removed and stored at -20°C until analysis. Analysis for plasma total protein, albumin and

inorganic phosphate was performed using a microcentrifugal analyser (Monarch 2000, Instrumentation laboratory, Warrington, Cheshire, UK).

2.2.3.4. Parasitological measurements

Weekly faecal samples were collected directly from the rectum of all the experimental animals for the determination of the concentration of nematode eggs in the faeces (FEC) by a modified flotation technique (Christie and Jackson, 1982) and were expressed as eggs per g of fresh faeces. Total worm egg output (TEO) was calculated using the measurements of feed intake, digestibility and FEC and an estimate of faecal dry matter using the formula $TEO = \text{Total daily faecal DM} \times \text{FEC} / \text{faecal DM \%}$; where: Total daily faecal DM = DM intake \times (1-DM digestibility). All animals were slaughtered on day 42. At the time of slaughter, worm recovery from the digesta and the intestinal mucosa of the small intestine following incubation in physiological saline at 37°C for 4 hours was performed on all infected animals. Worm burdens and the ratio of male to female adult worms were counted from a 1% aliquot and multiplied by 100 to give total worm numbers. Adult female fecundity was calculated by dividing the TEO on day 42 by the number of adult female worms present at slaughter and expressed as eggs per female per day).

2.2.3.5. Histochemical and immunohistochemical analysis

Samples of intestinal tissue from parasitised animals were removed at slaughter and preserved in either 4% paraformaldehyde (PF) in PBS solution for 6 hours at room temperature (Newlands *et al.*, 1984) or in zinc (Zn) salts

fixatives at room temperature for 24 hours (González *et al.*, 2001). After fixation, tissues were processed and embedded in paraffin; 5 µm sections were cut, mounted on slides and dried for 12 hours at 40 °C. In PF fixed sections, general histochemical characteristics were assessed following haematoxylin-eosin staining. MMC and eosinophils were enumerated following staining with toluidine blue (Enerback, 1966) and carbol-chromotrope (Lendrum, 1944), respectively. Periodic acid Schiffs (PAS) was employed to detect goblet cells in epithelium, and paneth cells in the crypts (Mantle and Allen, 1978). Alcian blue-PAS staining was used for identifying neutral and acidic mucins, as described previously (Newlands *et al.*, 1984). Stained cells were enumerated using a x 10 eye piece containing a calibrated graticule and a x 40 objective lens. For each graticule area, the top edge was placed parallel to the epithelial surface, such that the counts represented a mean of the epithelial and sub-epithelial regions. Zn salts fixed tissue sections were employed for immunostaining. The antibody signal was amplified with the Envision System Peroxidase using anti-mouse antibody (DAB) according to the manufacturer's instructions (Dako, Ltd, Ely, UK). Pan T cells were detected with anti-CD3 (AntiCD3, Dako, Ltd, Ely, UK) at a concentration of 1/100. Mouse monoclonal anti-ovine CD4 (Basel Institute of Immunology) at a concentration of 1/1000 was employed to detect ovine Th cells. Controls consisted of sections incubated without primary antibody. Stained cells were enumerated using Cell[^]F (Olympus imaging software, Copyright 1986-2006), the graticule was calibrated as part as the software. All cell counts were performed systematically in the epithelium and mucosa of the intestinal tissue and estimated from the mean of 10 graticules, and expressed as the number of cells/mm² of intestinal tissue.

2.2.4. Statistical Analysis

Analyses were performed using Genstat statistical software version 7.2 (Lawes Agricultural Trust, 2004) as a 2x2 factorial design with diet and infection as the factors unless otherwise stated. Prior to statistical analysis, FEC, TEO and worm burdens were log-transformed ($\log_{10}(x+1)$) and intestinal tissue cell concentrations were square-root transformed to remove positive skewness. All transformed data are presented as back-transformed means unless otherwise stated. Feed intake, liveweight, FEC, TEO and serum analysis were analysed using Restricted Maximum Likelihood (REML) with time included as a factor. Worm burdens, liveweight gain and intestinal tissue cell concentrations were analysed by using a general Analysis of Variance (ANOVA). For FEC, TEO, worm burden and cell concentrations, analysis was performed on data from infected animals only; consequently infection was removed as a factor.

2.3. RESULTS

2.3.1. Food composition

The two diets were of poor quality and were similar for most of the characteristics measured with the exception of grass hay containing slightly less crude protein and considerably less total phenolics and total CT (Table 2.1).

2.3.2. Food intake

The means for DM intake (DMI) per treatment are shown in Figure 2.2. Overall, for feed intake there was a diet x time interaction ($P < 0.001$) that reflected a mean feed intake of sainfoin of 1211 g/d that did not differ with time while intakes of grass fed animals increased from a mean of less than 500 g/d from day -14 to a mean greater than 1000 g/d from day 29. In addition, there tended to be an infection x diet x time interaction ($P = 0.069$) due to infection causing a 14% reduction of feed intake in grass-fed animals from day 39 compared with a 7% reduction observed in sainfoin-fed animals.

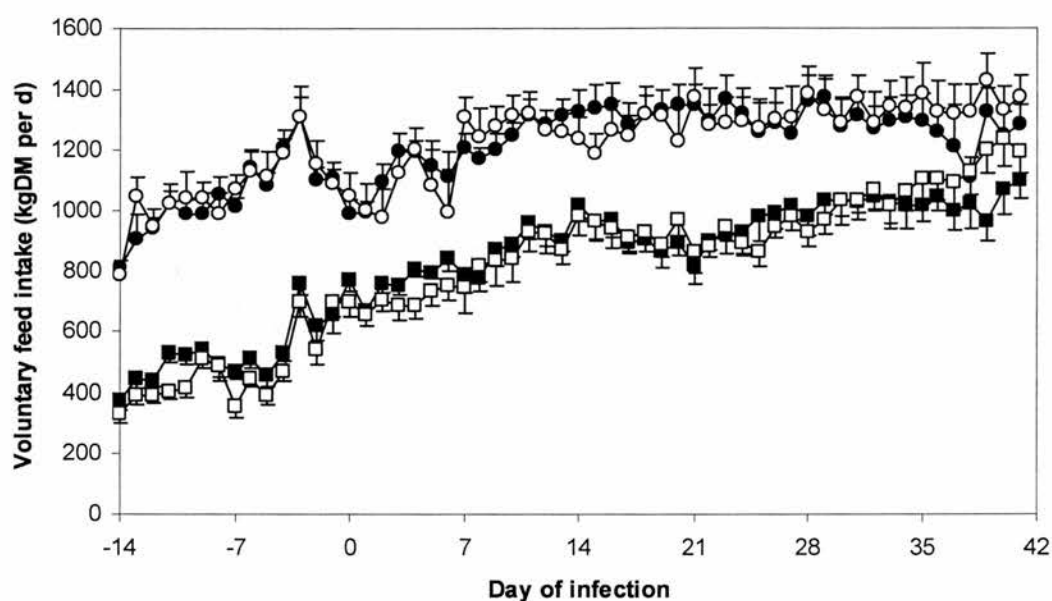


Figure 2.2. Means (\pm s.e.) of voluntary dry matter intake (kg/day) of animals that were either offered grass hay and infected with *Trichostrongylus colubriformis* (■), offered grass hay and not infected (□), offered sainfoin hay and similarly infected (●) or offered sainfoin and not infected (○).

2.3.3. Liveweight gains of the animals

Overall, for liveweight there was a diet \times time interaction that reflected a divergence in liveweight that increased with time. Liveweight gains during the infection period (from day 0 to day 42) were reduced by infection ($P=0.005$) and were lower in grass fed than in sainfoin fed animals ($P=0.002$). However, there was no significant infection \times diet interaction ($P=0.38$) with mean liveweight gains during the infection period being 10.6 ± 14.4 , 69.1 ± 11.4 , 75.2 ± 8.8 and 107.4 ± 22.8 g/day for G+, G-, S+ and S-groups, respectively.

2.3.4. Blood samples

Serum total protein concentration displayed a diet x time interaction ($P=0.05$) that was reflected in similar pre-infection concentrations of 68.3 ± 5.39 and 69.4 ± 5.28 g/l that were maintained in sainfoin fed animals but decreased in grass-fed animal from day 29, with mean concentrations on day 42 being 67.8 ± 6.73 and 64.1 ± 4.83 g/l for sainfoin and grass fed animals, respectively. For serum albumin, there were infection x time ($P=0.014$) and diet x time ($P=0.003$) interactions. The former was reflected in similar pre-infection albumin concentrations of 40.5 ± 2.02 and 40.1 ± 1.41 g/l for infected and non-infected animals, respectively, that were decreased at day 42 in infected but not in non-infected animals, *viz*, 35.3 ± 2.80 and 37.8 ± 2.32 g/l for infected and non-infected, respectively. The latter interaction was reflected in similar pre-infection concentrations of 40.4 ± 1.86 and 40.2 ± 1.65 g/l for grass and sainfoin fed animals that were decreased in grass fed animals from day 22, resulting in mean concentrations at day 42 of 35.8 ± 2.76 and 37.4 ± 2.74 g/l for grass- and sainfoin-fed animals, respectively.

Overall, serum phosphate concentrations were greater in sainfoin-fed animals, namely, 4.60 ± 1.60 and 5.16 ± 1.53 mg/dl for grass- and sainfoin-fed animals, respectively ($P<0.001$). In addition, there was an infection x time interaction ($P<0.001$) that reflected similar concentrations at day -14 of 4.14 ± 1.13 and 4.48 ± 0.89 mg/dl in infected and non-infected animals, respectively, that were decreased by a mean of 30% in infected animals from day 15, with concentrations at day 42 being 3.34 ± 0.94 and 5.59 ± 1.28 mg/dl for infected and non-infected animals, respectively.

2.3.5. Parasitological measurements

Back-transformed faecal nematode egg counts (FEC) are given in Figure 2.3. Overall, there was an effect of time and diet ($P < 0.001$ for both) with an increase in the concentration of eggs with time and greater concentrations observed in grass- compared with sainfoin-fed animals. Due to the differences in feed intake between sainfoin and grass fed animals, total daily nematode egg output (TEO) was estimated. For TEO, egg output increased with time ($P < 0.001$) from 526 017 on day 21 to 1.1×10^6 on day 42 but was not affected by diet ($P = 0.46$).

Worm burdens recovered from the small intestine are shown in Table 2.2. Overall, there was no effect of diet on the number of juvenile worms ($P = 0.43$), adult males ($P = 0.46$), adult female ($P = 0.57$), total adult burdens ($P = 0.48$), total worm burdens ($P = 0.58$) or female adult fecundity ($P = 0.27$).

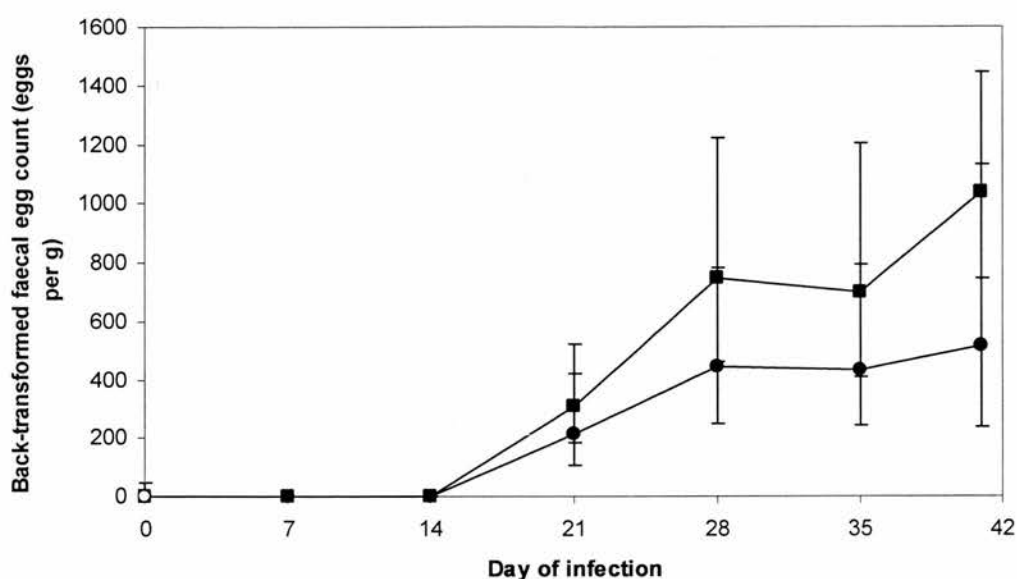


Figure 2.3. Mean backtransformed faecal egg counts (FEC; eggs/g) for parasitised animals that were offered either grass hay (■) or sainfoin hay (●). Error bars represent 95% confidence intervals.

Table 2.2. Arithmetic mean \pm s.e. for numbers of worms and adult female fecundity (eggs/female/day) of *Trichostrongylus colubriformis* worms that were retrieved from animals after 42 d of infection and offered either grass hay or sainfoin hay.

	Grass Hay	Sainfoin Hay	s.e.	
Juvenile	6 525	5 625	-	$P=0.43$
	(4 000 - 10 200)	(3 900 - 9 300)		
Adult female	19 512	21 262	-	$P=0.57$
	(11 400 - 25 700)	(16 100 - 37 100)		
Total adult female	36 288	40 325	-	$P=0.48$
	(18 600 - 50 000)	(28 200 - 68 300)		
Total	42 812	45 950	-	$P=0.58$
	(22 600 - 55 300)	(33 100 - 74 700)		
Adult female fecundity	73.6	57.3	10.08	$P=0.27$
	(25.4 - 111.6)	(16.8 - 98.5)		

(Lower (25%) and upper (75%) quartile threshold values are given in parenthesis. Statistical comparisons for worm numbers were performed on log10 transformed data. No significant differences for any parameter were detected)

2.3.6. Intestinal tissue cell counts

Numbers of immunological cells per mm² of intestinal tissue in parasitised animals at slaughter are given in Table 2.3. Compared to their grass fed counterparts, intestinal tissues of sainfoin fed animals had greater concentrations of both Pan T cells ($P=0.01$), MMC ($P=0.04$) and eosinophils ($P<0.001$). Grass and sainfoin fed animals had similar concentration of T helper cells ($P=0.25$), Paneth cells ($P=0.15$) and goblet cells in both the epithelium ($P=0.26$) and crypt ($P=0.22$). Of the goblet cells, there was no difference between diets in the concentration of cells containing neutral mucin ($P=0.54$). However, there was a tendency for a greater concentration of goblet cells containing acidic mucins (alcian blue positive) in grass fed animals ($P=0.13$), with these alcian blue positive cells comprising 39 and 25% of the total number of goblet cells for grass- and sainfoin-fed animals, respectively Figure 2.4 (A-F).

Table 2.3. Arithmetic mean \pm s.e. concentrations (cells per mm²) of T cell subsets, mucosal mast cells (MMC), eosinophils, goblet cells, paneth cells and mucin-producing cells in intestinal tissues retrieved from lambs after 42 days of infection with *Trichostrongylus colubriformis* while offered either grass or sainfoin hay.

	Pan T cells	T helper cells	MMC	Eosinophils	Goblet cells		Paneth cells	Mucin-producing cells		
					Epithelium	Crypts		Neutral	Mixture	Acid
Grass Hay	579 \pm 155 (471-667.1)	111 \pm 80.4 (18-183)	24.7 \pm 15.8 (8.6-33.7)	11.4 \pm 7.9 (3.8-17.9)	37.9 \pm 32.4 (9.8-61.6)	104 \pm 58.3 (70-143)	87.7 \pm 48.4 (52.7-132)	14.6 \pm 9.8 (7.9-19.4)	8.2 \pm 5.2 (4.8-10.1)	13.5 \pm 7.9 (7.0-22.4)
Sainfoin Hay	741 \pm 79.7 (675-822)	153 \pm 70.9 (104-167)	44.4 \pm 20.1 (26.4-58.3)	41 \pm 16.1 (33.6-53.5)	54.6 \pm 13.2 (43.5-63.6)	136 \pm 18.2 (121-149)	122 \pm 21.3 (104-144)	16.7 \pm 7.3 (11-21.5)	9.1 \pm 3.8 (6.0-11.9)	8.2 \pm 4.4 (4.6-12.3)
	P=0.01	NS	P=0.04	P<0.001	NS	NS	NS	NS	NS	NS

(Lower (25%) and upper (75%) quartile thresholds are given in parenthesis. Statistical comparisons were made on square-root transformed data.)

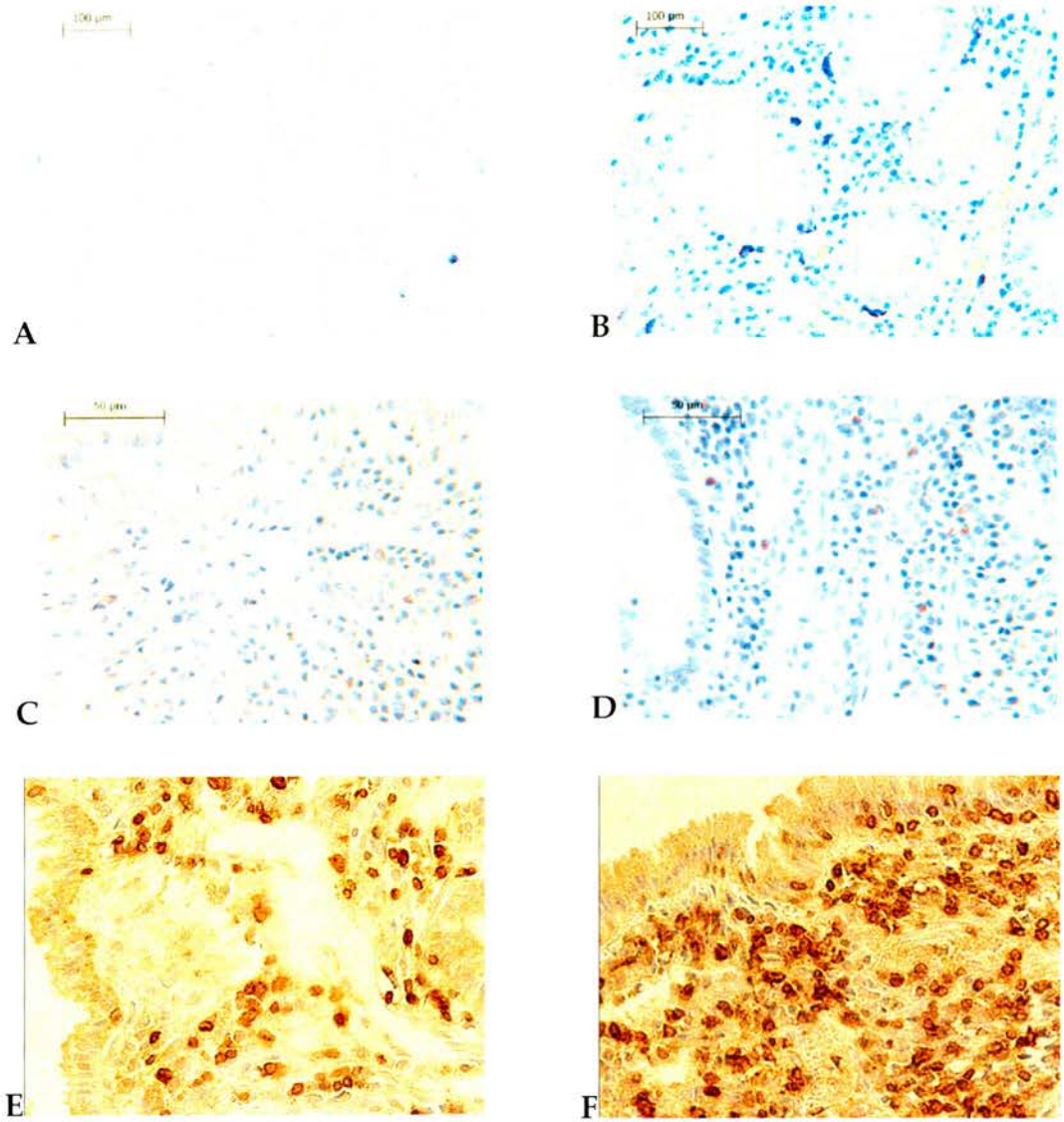


Figure 2.4. A-F. Photographs (40x) of mucosal mast cells (A, B), eosinophils (C, D) and Pan T cells (E, F) in sections from intestines of hay fed animals (A, C, E) compared to intestine samples of animals fed with sainfoin (B, D, F). Significantly higher numbers of cells were observed in the sainfoin fed animals.

2.4. DISCUSSION

In the present study, attempts were made to address the anthelmintic properties of sainfoin hay, and investigate possible modes of action. Although feeding sainfoin did not have significant effects on worm burdens or total egg output, an intriguing finding was that sainfoin enhanced some local cellular responses associated with GIN immunity (Miller, 1984). This result suggests that components within sainfoin are able to enhance the normal local pathological and/or immune responses which occur during infection, either directly, or through improved nutrition. It is not possible to be certain of the mechanism because although the grass and sainfoin diet were chosen to have similar protein and energy content, due to differences in intake, the mean intake of protein was one third higher in the sainfoin animals compared with the grass-fed animals (117 *vs.* 77g crude protein-CP).

In contrast to the present results, studies in adult goats using 5% Q (Paolini *et al.*, 2003a) or sainfoin hay containing 2.7% CT (Paolini *et al.*, 2005b) showed a clear effect of sainfoin hay consumption on *T. colubriformis* egg excretion and worm burden, although no such effects were observed for *H. contortus* infections when sainfoin hay containing 3.2% was offered (Paolini *et al.*, 2005a). Furthermore, using naïve lambs infected with *T. colubriformis*, Athanasiadou *et al.* (2005a) found animals offered lucerne hay had a significantly higher egg output than those animals receiving sainfoin hay. The studies of Heckendorn *et al.* (2006, 2007) were the first to find dramatic results of sainfoin on *H. contortus* in sheep and some minor effects on *Cooperia curticei*. In the first study, Heckendorn *et al.* (2006) reported a reduction in FECDM (faecal egg count on a dry matter basis) by 58% in the

group offered sainfoin hay that contained 6.1% CT and a 48% reduction in the FECDM in the group offered sainfoin silage containing 4.2% CT when compared to the FECDM of the group offered control diets containing 0.1% CT. In the second study, Heckendorn *et al.* (2007) found a significant reduction in the total daily faecal egg output for *H. contortus* of 89% for animals fed with chicory containing 0.3% CT and 63% for those fed with birdsfoot trefoil and sainfoin containing 1.5% and 2.6% CT, respectively, indicating that the effect found in chicory could be due to a PSM other than CT. However, in the present study, worm burdens and fecundity were not affected by the feeding treatment of the animals in the time frame (6 weeks) investigated. It appears from the literature that differences related to the source (plant species), CT content, changes in CT content within forage species, species of the parasite and of the host, and time of exposure to both parasites and PSM are all factors that may influence the anthelmintic effect. Perhaps the latter aspect is of particular relevance in the present study, where animals were challenged with parasites for a relatively short (6 weeks) period. Previous studies have shown that *Trichostrongylus* populations are not subject to obvious regulation, including falls in egg counts or worm burdens, until after 6 weeks of exposure to infection (Seaton *et al.*, 1989a; Bown *et al.*, 1991; Coop *et al.*, 1995). While sainfoin induced increases in local inflammatory and immune cell populations, these were clearly not sufficient to result in a reduction in parasite worm burdens or egg production. However, since worm immunity is a dynamic and complex process, it is possible that a longer exposure to the parasites and sainfoin may have resulted in a more rapid, or of a greater magnitude, immune-mediated response.

There is much evidence for the important role of MMC and epithelial globule leukocytes in the local immune response of rodents and sheep to parasites (Miller, 1984; Huntley, 1992; Balic *et al.*, 2000). However, the ability of PSM to influence these inflammatory responses to GIN has not previously received much attention. In previous work by Paolini *et al.* (2003a), drenching goats infected with *H. contortus* with quebracho, did not modify the eosinophils, MMC or globule leukocytes in the abomasal tissue. However, the administration of the extract was relatively short term. In a different study in growing lambs, Tzamaloukas *et al.* (2006) showed that animals grazing sulla or chicory had an enhanced immunity against *T. circumcincta*, with elevated counts for globule leukocytes and MMC in abomasal tissues, but no differences for eosinophils. However, the results found in the previous two studies are difficult to compare because of the differences in host species, parasite species, source of PSM and experimental design used.

Both eosinophils and mucin-producing goblet cells have also been implicated in the protective effector immune responses against GIN. Eosinophils may damage or kill GIN larvae *in vivo* (Balic *et al.*, 2006), and goblet cell hyperplasia is a characteristic feature of infection (Ishikawa *et al.*, 1997). In addition to the increase in goblet cells, studies in sheep have shown that the quality of mucins changes from an essentially neutral to acidic composition during the acquisition of immunity (Newlands *et al.*, 1990). These changes may enhance the exclusion of larvae, by trapping and prevent them from entering the crypt regions (Miller *et al.*, 1981), possibly by increasing the viscosity or physical nature of the mucin, or enhanced binding of specific anti-worm proteins such as IgA antibodies or intelectin (Pemberton *et al.*, 2004). However, there appeared to be no quantitative changes in these cells between sainfoin and grass fed animals in the time frame investigated.

Previous work has shown that goblet cell hyperplasia is controlled by T cells (Khan and Collins, 2004), and a significant increase in Pan T cells was demonstrated in the lamina propria in this study. While T helper cells are known to be principally involved (Khan and Collins, 2004), the identity and proportions of individual T cell sub-populations were not determined in this work.

A range of nutritional components are known to influence the cells associated with the acquisition and expression of immunity including protein and energy (Coop and Kyriazakis, 1999). Both hays were considered in terms of protein and energy as being relatively similar but poor nutrient sources (see Table 2.1.). However, the greater feed consumption of sainfoin makes the possible direct nutritional effect and that of a direct action of PSM's difficult to separate. Post-ruminal infusions of casein supplying 50g of metabolisable protein (MP) per day in addition to a basal diet of approximately 50g MP per day have been shown to enhance the rate of acquisition of immunity (Bown *et al.*, 1991). Unfortunately Bown *et al.* (1991) did not monitor tissue cell counts, however, these authors observed no difference in worm burden after six weeks but reduced worm burdens after 12 weeks of infection in casein supplemented animals of a comparable age and infected with the same nematode species as the current study. In the present study during the course of the infection, sainfoin fed animals consumed a mean of 117g CP per day in comparison to the 77g CP consumed by their grass-fed counterparts. Therefore, it may be possible that the enhanced immunological activity observed in the tissue retrieved from sainfoin fed animals was a reflection of the greater nutrient supply allowing an earlier acquisition of inflammatory responses that are associated with immunity (Miller, 1984). Comparable to the aforementioned results of Bown

et al. (1991), the expression of such immune mechanisms, in terms of reduced parasite burden and/or parasite fecundity, would not be expected given the short time-frame of infection.

Despite this difference in total nutrient intake, there is a plethora of evidence to support the immuno-stimulatory properties of PSM. In particular, lectins are PSM that have known immunostimulatory properties and have been widely employed to non-specifically activate T cells inducing T cell proliferation (Kay, 1991). Although lectins have been isolated from sainfoin (Young *et al.*, 1982; Kouchalakos *et al.*, 1984) the properties of this protein have not yet been studied. Clearly, further fractionation studies will be required to attempt to identify which components in sainfoin are responsible for the local cellular responses observed, - a challenging task given the huge range of proteins and bioactive molecules that will be present.

In summary, these results support the notion that feeding sainfoin hay may act to enhance the inflammatory and immune cell response to GIN. From the current study it is unclear if this is due to enhanced nutrition supplied by the sainfoin or if it is due to the direct immuno-stimulatory properties of PSM. However, at this relatively early stage in the development of acquired immunity there did not appear to be any significant reductions in worm burden or total egg output. Nevertheless, the present study adds to the increasing evidence that dietary factors are able to influence the host's local immune reaction to intestinal parasites, although further studies would be required to define the active components in sainfoin and to elucidate their mode of action.

IN VITRO SCREENING OF PLANT LECTINS AND TROPICAL PLANTS EXTRACTS FOR ANTHELMINTIC PROPERTIES

3.1. INTRODUCTION

Plant lectins are proteins/glycoproteins known for their ability to bind specific carbohydrates and have been widely used as immunostimulants for T cell activation in artificial systems. Their concentrations vary with plant species, cultivar and regions of the plant, being found mainly in the seeds (Grant, 1999). Like other PSM, lectins are not involved in the primary metabolism of the plant. However, there are several possible functions that have been suggested for these compounds, the main one being plant defence (Peumans and Van Damme, 1995), since lectins have been found to be toxic to plant nematodes (Gaofu *et al.*, 2008), insects (Habibi *et al.*, 1993) and birds (Jayne-Williams and Burgess, 1974).

Another important feature of plant lectins is the stability during passage through the gastrointestinal tract of animals. Nakata and Kimura (1985) showed that 90% of the Con A was recovered unaltered 4 days later in the faeces of rats consuming 0.3 to 0.5% of this lectin in the diet. Also, Pusztai *et al.* (1990) showed that 100% of the PHA administered intragastrically to rats, remained immunochemically intact in the small intestine after 1 h and it was found binding to the brush border on first exposure. Finally, Oliveira *et al.* (1994) detected Con A lectin fully active against rabbit erythrocytes in the

faeces of rats fed lectin-containing diets. Specifically in ruminants, Baintner *et al.* (1993a) studied the degradation *in vitro* of 15 different plant lectins in rumen liquor. They found that PHA was highly resistant to ruminal proteolysis. In a different study (Baintner *et al.*, 1993b) defatted raw soyabean flour was given to cannulated sheep, as a source of soyabean lectin. The lectin appeared in the duodenum within one hour and reached the terminal ileum and disappeared within 24 hours. The lectin was found to bind the intestinal surface and these authors found that the anti-nutritional proteins were not effectively degraded in the forestomachs.

The literature also shows the uses plant lectins have as potent immunogens, both *in vitro* and *in vivo* (Kilpatrick, 1999). Several authors have shown how animals fed with diets containing lectins, or dosed with lectins, had an enhanced immune response as indicated by high titres of circulating IgG to the lectin (Pusztai, 1991; Lavelle *et al.*, 2000), or through stimulated lymphocyte production (Gamble, 1966), among others. These features of plant lectins, stability in the gastrointestinal gut and the fact that they are potent immunogens, were essential to the decision to study plant lectins for possible effect on GIN.

The previous chapter examined the effect that feeding a PSM-rich plant, sainfoin, had on GIN. Although treated animals had a lower FEC at specific times of the trial, no negative effect was found on LWG. Moreover, animals consuming sainfoin had increased numbers of MMC, eosinophils, goblet cells, Pan T cells and T helper cells, suggesting that this PSM-rich plant was enhancing the immune response against infection with *T. colubriformis*. However, PSM other than tannins have not been studied extensively, raising

the question of how much of this increase in immunity is a result of the intake of other immunostimulatory plant compounds such as lectins.

The aim of this Chapter was to use a number of different *in vitro* assays to study the potential direct effects of plant lectins and four forages used for livestock feeding in tropical and subtropical countries. The studies aimed to make extensive use of a sensitive bioassay, the larval feeding inhibition assay (LFIA). A further aim of the study was to attempt to identify the possible site(s) of action of the lectins on the larvae. The assay used in this study 'LFIA' was originally developed for studies on anthelmintic resistance (Álvarez-Sánchez *et al.*, 2005) but the LFIA can also be employed as a powerful tool in the search for new compounds with anthelmintic potential (Gordon *et al.*, 2007). The studies in this chapter have used this methodology for the first time to examine the anthelmintic potential of plant lectins.

The key PSM tested in this study are plant lectins, defined as carbohydrate-binding proteins/glycoproteins which can be separated into 8 groups according to their general carbohydrate-binding specificity: complex, fucose, galactose, N - acetylglucosamine, mannose, mannose / glucose, mannose / maltose, sialic acid (Grant, 1999). In these preliminary studies a selection of lectins, chosen on the basis of availability and cost, were tested for their direct anthelmintic effects on the free living stages of trichostrongylid GIN. Concanavalin A (Con A) from *Canavalia ensiformis* (jack bean) has been shown to have a high affinity for glucose / mannose (Glu / Man) (Sharma and Surolia 1997). WGA is described by Chrispeels and Raikhel (1991) as a seed *Gramineae* lectin which specifically binds to the sugar N-acetylglucosamine (GlcNAc). PHA lectins (PHA-E and PHA-L) recognize terminal galactose residues on complex glycans of mammalian glycoproteins. Fetuin (FET),

which is a large complex of carbohydrates capable of binding to a wide range of lectins. FET was used as the inhibitor of PHA-E3L (E3L), in order to establish if the action of the plant lectin or plant extract is due to the lectin *per se* or if it is due to a different compound. FET was identified by Pedersen (1944) from the Institute of Physical Chemistry of Uppsala, during some studies of fractionation of calf serum with ammonium sulphate and making comparisons with cow serum, this new globulin was found and proposed to be termed 'fetuin', derived from *foetus*. For testing different plant compounds these sugars are an important tool for separating possible effects, for example a specific binding sugar can inhibit the action of the lectin. Thus, the addition of a specific or complex sugar to the assay can confirm that the effect using a plant extract is likely to involve a lectin. Similar approaches have also been made with other bioactive PSM, using polyethylene glycol (PEG), polyvinyl pyrrolidone (PVP) or polyvinyl polypyrrolidone (PVPP) as inhibitors for CT, also called tannin-complexing agents (Villalba *et al.*, 2002; Paolini *et al.*, 2004a; Barrau *et al.*, 2005; Hoste *et al.*, 2006; Brunet *et al.*, 2008; Waghorn, 2008).

Since FET is a large and complex sugar with multiple lectin specificities, its addition to complex plant extracts where the lectin component and its sugar specificity is unknown provides the best chance of inhibiting any unknown lectin component. Inhibition of larval feeding behaviour may be caused by a range of plant secondary metabolites including CT and lectins. To investigate the relative contributions made by CT and/or lectins in the plant extract studies using the LFIA, the extracts were tested in the presence of PEG which binds and inactivates CT by leaving them unable to bind to parasite proteins, as well as the addition of FET. The extent to which these inhibitors restored feeding activity would provide information on the relative importance of the CT and lectins as modifiers of larval feeding behaviour.

Beside lectins, the tropical plants tested in the current study were *Gliricidia sepium*, *Leucaena leucocephala*, *Azadiractha indica*, *Morus alba* and *Trichanthera gigantea*. These plants were chosen because they are normally used in the tropics and subtropics, for feeding livestock on farms. *G. sepium* and *L. leucocephala* are leguminous and PSM-rich plants (Seresinhe and Iben, 2003; Rojas *et al.*, 2006), that were chosen as potential anthelmintic plants. In similar conditions, *A. indica* even though it is not a legume, is a plant widely known for its medicinal and anthelmintic properties. In contrast to these three species, *M. alba* and *T. gigantea* were chosen as controls because are not known as PSM-rich plants, but are highly nutritious and widely used as feed and supplementary feed for livestock.

Different studies show *G. sepium* and *L. leucocephala* as two of the most promising fodder trees with potential to be incorporated into silvopastoral systems in the northern part of Mexico (Jiménez-Ferrer *et al.*, 2008) and used for restricted grazing in Venezuela (Combellas *et al.*, 1996). In different studies all these species were shown to be important species for livestock simply because of their nutritional quality and mineral content. *Morus alba*, also called Mulberry, which was originally used for feeding silkworms, has been shown to be a high quality forage for small ruminants (Doran *et al.*, 2007).

3.2. MATERIALS AND METHODS

3.2.1. Experiment 1: Larval feeding inhibition assays (LFIA) using plant lectins

3.2.1.1. Lectin isolation

Kidney bean (*Phaseolus vulgaris*) albumin (lectin-rich) proteins were isolated at the Rowett Research Institute according to the methodology described by Pusztai *et al.* (1990) and Grant (1999). Beans were ground using a hammer mill fitted with a 1 mm mesh. The meal (100g) was mixed with 1 litre of 0.2M sodium acetate pH 5.0 buffer and left stirring at 4°C overnight (approximately 20 hours). Large particulate matter was allowed to settle out. The upper liquid was decanted off, centrifuged at 48000g for 15 minutes and the supernatant poured off and filtered through glass wool.

The clear supernatant was then mixed with ammonium sulphate to 80% saturation (608 g ammonium sulphate/litre), the proteins allowed to precipitate (no stirring) at 4°C overnight (approximately 20 hours). The clear upper liquid was decanted off and discarded. The remaining liquid/precipitated proteins were centrifuged at 48000g for 15 minutes. The supernatant was discarded. The pellet was re-suspended in water (200-300 ml), the pH of the solution adjusted to 7.0 and then dialysed (three changes) against distilled water (4 litre). After dialysis, the pH of the solution was checked, the solution centrifuged (if necessary) and the supernatant lyophilized. The freeze material (Kidney bean albumin; KBA) contained on

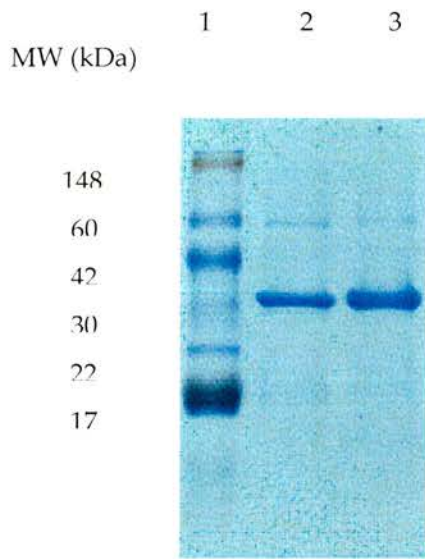
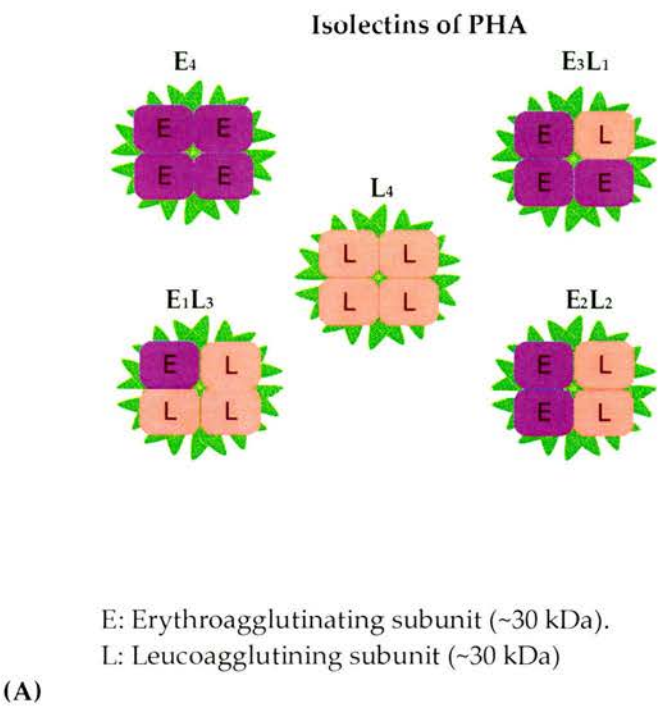
average 250-400g lectin / kg dry weight, as estimated by lectin-specific ELISA done at the Rowett Research Institute.

3.2.1.2. Lectin selection

A first screen was performed using 13 different lectins (Table 3.1), and distilled water as a control. Kidney bean lectins E3L, E2L2 and L4 (Figure 3.1) were isolated at the Rowett Research Institute by affinity and ion-exchange chromatography procedures (Pusztai *et al.*, 1990), E4 and EL3 were not used because of its low availability for this study. Other lectins were purchased from Sigma-Aldrich, EY Laboratories Inc or Vector Laboratories Ltd. The extract powder was weighed and mixed with distilled water in universals using a vortex, to obtain a 1000 µg/ml solution. This concentration was chosen as the highest one based on *in vivo* experiments at the Rowett Research Institute, and was used as a stock solution from which the dilutions were made (Grant, 2009).

Table 3.1. Lectins tested in the first screen for anthelmintic properties using the larval feeding inhibition assay.

Lectin Name	Abbreviation	Plant/Source
Jacalin (Jack fruit agglutinin)	JAC	Jack fruit (<i>Artocarpus integrifolia</i>)
Concanavalin A (Jack bean lectin)	Con A	Jack bean (<i>Canavalia ensiformis</i>)
Phytohemagglutinin E2L2	PHA-E2L2	Kidney bean (<i>Phaseolus vulgaris</i>)
Phytohemagglutinin L4	PHA-L4	
Phytohemagglutinin E3L	PHA-E3L	
Kidney bean albumin	KBA	Kidney bean (<i>Phaseolus vulgaris</i>)
<i>Robinia pseudoacacia</i> agglutinin	RPA	Locust tree (<i>Robinia pseudoacacia</i>)
<i>Maackia amurensis</i> lectin	MAA	Amur maackia (<i>Maackia murensis</i>)
<i>Maclura pomifera</i> agglutinin	MPA	Osage orange (<i>Maclura pomifera</i>)
<i>Dolichos biflorus</i> agglutinin	DBA	Horse gram (<i>Dolichos biflorus</i>)
Wheat germ agglutinin	WGA	Wheat (<i>Triticum vulgare</i>)
<i>Galanthus nivalis</i> agglutinin	GNA	Snowdrop (<i>Galanthus nivalis</i>)



1. Standard. 2. Kidney bean lectin L4. 3. Kidney bean lectin E₂L₂.
(B)

Figure 3.1. (A) Diagram of tetrameric composition of kidney bean isolectins and (B) SDS-PAGE showing subunits of PHA. SDS-PAGE was carried out under denaturing conditions by standard procedures. This was performed by Dr. Michelle Duncan at the Rowett Research Institute.

3.2.1.3. Parasite isolates

First stage larvae of the most economically important ovine GIN *H. contortus*, *T. colubriformis* and *T. circumcincta* were used in the studies. These larvae were obtained from faeces collected from monospecifically infected male lambs maintained at Moredun Institute. Each lamb was fitted with a harness and collecting bag to enable faeces to be collected. Faecal material was collected over a 2 hour period and dispersed in tap water (10mls water/gm faeces) the eggs and fine faecal debris were recovered by washing the faecal suspension over a range of sieves (400, 150, 64 and 37 μm sieves). The retentate from the 37 μm sieve was washed into polyallomer centrifuge tubes (Beckmann Ltd) and following centrifugal sedimentation of the eggs and debris, the eggs were recovered by centrifugal flotation in saturated NaCl solution (S.G. 1.2). Clean eggs were incubated for 24 hours at 25 °C and hatched to obtain the L1 which were recovered by baermannization. The concentration of L1 was adjusted to provide 100 L1/100 μl .

3.2.1.4. Larval feeding inhibition assay (LFIA)

This LFIA bioassay was used in this study in order to investigate the effect of plant lectins on the feeding of L1. The larvae were incubated in the lectins (1000 $\mu\text{g/ml}$) for 2 hours at 25 °C and then 10 μl of a nutritive medium was added and incubated for a further 18 h, at the same temperature. All concentrations and controls were performed using 3 replicates. After this time fed larvae were counted (observation of intestinal fluorescence) using a fluorescence inverted microscope fitted with a blue filter (wavelength 470 nm) (Álvarez-Sánchez *et al.*, 2005).

The nutritive medium used for the LFIA was FITC (Sigma Chemical) labelled *Escherichia coli*. This medium was prepared using a modified method from Geary *et al.* (1993), one millilitre of a concentrated solution of lyophilised *E. coli* (Sigma, UK) was mixed with 0.2 mg of FITC (Sigma Chemical) in 1 ml of modified bicarbonate buffer (2.66 g/L NaHCO₃, 1.96 g/L Na₂CO₃, and 1.5 g/L NaCl, at pH 7.4). After a 2 h-incubation period and four phosphated-buffered saline (PBS, pH 7.4) washes the labelled *E. coli* final working-concentration was adjusted to 11,250 µg/ml (Álvarez-Sánchez *et al.*, 2005).

After the primary screening, the three most potent lectins (100% unfed larvae) were chosen and used in further assays, in which the range of concentrations used was from 0.5 to 500 µg/ml, based on normal dilutions used in the laboratory for previous experiments with plant extracts (Gordon *et al.*, 2007).

3.2.1.5. LFIA using lectin inhibitors

In order to confirm that any anthelmintic effect was specifically due to lectin activity, the LFIA were repeated with the addition of the specific sugars or inhibitors, using *H. contortus* larvae. The inhibitors were used in two different concentrations with their lectin. For Con A, Glu/Man was used at 0.03 and 0.06M, for WGA GlcNAc was used at 0.06M and 0.12M and for PHA-E3L, FET was used in two ratios of 1:3 and 1:10 [w:w lectin:fetuin] (Grant, 1999). All of the inhibitors were tested alone at their working concentrations in two replicates, to measure any direct effects they could

have on the larvae. After this the lowest concentration of the inhibitor with low or no effect on the larvae was selected for inhibition studies in the LFIA.

3.2.1.6. Binding sites of the lectins to the larvae

Two methodologies were used for investigating the binding sites of the lectins:

- 1. LFIA:** assays were conducted using *T. circumcincta* L1 incubated in Biotin labelled WGA, Con A and PHA and compared with control L1's incubated in water, all fed with FITC labelled *E. coli*. Based on previous results with LFIA, the concentrations used for the biotinylated lectins were: for WGA and Con A 20, 200 and 500 µg/ml and for PHA 1, 10 and 500 µg/ml. After overnight incubation at 25 °C the larvae were washed twice (6000 rpm x 3 min) and streptavidin-alexafluor® 555 conjugate (catalog no. S21381, Invitrogen™) (streptavidin-af) was added (1/100) and left for 2 hours, after this final incubation the larvae were washed and centrifuged three times (6000 rpm x 3 min) and checked with a stereo microscope (400x). FITC labelled Con A, WGA and PHA were also tested by incubating them at 25 °C overnight with *T. circumcincta* L1. After overnight incubation at 25 °C the larvae were washed and centrifuged twice (6000 rpm x 3 min), left in water and checked with a stereo microscope (400x).
- 2. Immunohistochemistry on worm sections:** Using a modified methodology from Clark (2006), L1 and exsheathed L3 of *H. contortus* were fixed by immersion in the following fixatives: **(1)** Zn salts (ZSF), 0.5% (w/v) Zn acetate, 0.5% (w/v) ZnCl₂, 0.05% (w/v) Calcium acetate,

0.1 M Tris, (pH 7-7.4), **(2)** 4% (v/v) paraformaldehyde (in PBS) or Modified Bouin's (2.5% glacial acetic acid; 5% (v/v), formaldehyde 37%; 92.5% (v/v) saturated aqueous picric acid) solution for 24-28 h at 4 °C. Using centrifugation (10000 rpm) the larvae were washed three times and pelleted using agarose (10% (w/v) gelatine). These pellets were fixed in 70% (v/v) ethanol for a further 48 hours at 4 °C. Following dehydration using graded alcohols, samples were embedded in paraffin wax at 56 °C. Sections were cut as required for immunohistochemistry analysis. For the FITC labelled lectins the different treatments included were control (no treatment), FITC Con A (20 µg/ml), FITC PHA (20 µg/ml) and FITC WGA (20 µg/ml).

3.2.2. Experiment 2: Larval exsheathment assay (LEA) using plant lectins

3.2.2.1. LEA

To study the effect of plant lectins on the process of exsheathment of L3 larvae of *T. circumcincta* the larval artificial exsheathment assay was developed following a similar methodology to that described by Brunet *et al.* (2007) and using the plant lectins used for the LFIA, Con A, WGA and PHA-E3L. For the assay L3 larvae were obtained from a donor infected with a pure isolate of *T. circumcincta*. Ensheathed larvae were incubated in different plant lectin solutions using the concentration of 1000 µg/ml for 3, 6 and 24 hours. After this incubation, the larvae were washed and centrifuged 3 times in phosphate buffer solution (PBS; 0.1 M phosphate, 0.005 M NaCl, pH 7.2) and

then placed into a solution of sodium hypochloride (2% w/v) and sodium chloride (16.5% w/v) diluted in 1 to 300 in PBS (Milton solution) to cause exsheathment. The kinetics of exsheathment was observed at different times (10, 20, 30, 40, 50, and 60 min) after contact with the exsheathment solution, using microscope magnification (200x). PBS was used for negative controls.

3.2.3. Experiment 3: Larval feeding inhibition assays (LFIA) and exsheathment assays using tropical plant aqueous extracts

3.2.3.1. Plant aqueous extracts

The plant species *Gliricidia sepium* (GLI), *Leucaena leucocephala* (LEU), *Morus alba* (MOR), *Azadiractha indica* (NEE) and *Trichanthera gigantea* (NAC) were collected in Venezuela. From oven dried and ground foliage, extracts were made fresh for each assay. A weighed amount of plant powder was soaked in distilled water and vortexed for 3 min. The stock solution was centrifuged and sieved (using disposable syringe filter, 0.2µm) prior to being used in the larval feeding inhibition assay. From the stock solution all the lower concentrations were prepared. The parasite chosen for these assays was *H. contortus* because it is the most common GIN in tropical conditions. *H. contortus* L1 were incubated with the different plant extracts; concentrations ranging from 0.2 to 10 mg/ml were generally used. However, because of its potent effect GLI was tested in concentrations that ranged from 0.005 to 10 mg/ml. Distilled water was used to provide control data. The methodology used for the LFIA was the same as that described for

Experiment 1. The inhibitors used in the case of plant extracts were PEG, for testing the effect of tannins in the extracts on the feeding of the larvae, and FET for the study of the possible effect of lectins in the plant extracts. All assays were performed in triplicates

3.2.4. Statistical Analysis

The percentage of larvae that fed at different plant concentrations was used to calculate the inhibitory concentration IC₅₀ estimates (the concentration of drug needed to inhibit worm feeding). In this study IC₅₀ was used for the compound concentration that inhibits the feeding of 50% of the larva) using Probit analysis (Minitab, release 13.1). Comparisons of IC₅₀ between lectins and between plant extracts were performed by Chi-square (Statistix 8.0, 2005).

3.3. RESULTS

3.3.1. Experiment 1: LFIA using plant lectins

All larvae failed to feed when treated with E2L2, MPA, DBA, E3L, WGA or Con A at a concentration of 1000 µg/ml (Table 3.2). In contrast, PHA-L4, RPA, MAA, KTI and GNA had much less effect. With these lectins, the majority of larvae (on average 70%) continued to feed. E3L, WGA and Con A were selected for further testing against the three species of GIN, based on their ready availability.

Observations on larvae incubated in E3L and Con A at the higher concentration of 1000 µg/ml, indicated that they were alive but unable to feed on the fluorescent *E. coli*. WGA appeared to have the most profound effect, as a concentration of 1000 µg/ml, was sufficient to kill 100% of larvae with evidence of damage to their cuticles which appeared to be swollen with the outer tegument of the cuticle being blistered with an obvious loss of integrity (Figure 3.2).

The IC₅₀ values for each lectin and parasite species are shown in Table 3.3. The most effective lectin with the lowest IC₅₀ value was E3L ($P=0.0006$) compared to Con A and WGA, that were effective at higher concentrations and *T. colubriformis* appeared to be the most susceptible ($P=0.07$) of the three species tested.

Table 3.2. Results obtained with lectins tested in the first screen using the larval feeding inhibition assay.

Lectin Name	Solubility	Results (% of fed larvae)
Jacalin (Jack fruit agglutinin)-JAC	Poor	-
Concanavalin A (Con A or Jack bean lectin)-Con A	Good	0
Phytohaemagglutinin E3L-PHA-E3L	Good	0
Phytohaemagglutinin E2L2-PHA-E2L2	Good	0
<i>Maclura pomifera</i> agglutinin-MPA	Good	0
<i>Dolichos biflorus</i> agglutinin-DBA	Good	0
Wheatgerm agglutinin-WGA	Good	0
Kidney bean albumin-KBA	Poor	24
Soy bean kunitz inhibitor-KTI	Poor	28
Phytohaemagglutinin L4-PHA-L4	Good	81
Snowdrop lectin (<i>Galanthus nivalis</i> agglutinin)-GNA	Good	83
<i>Robinia pseudoacacia</i> agglutinin-RPA	Good	97.5
<i>Maackia amurensis</i> lectin-MAA	Good	98

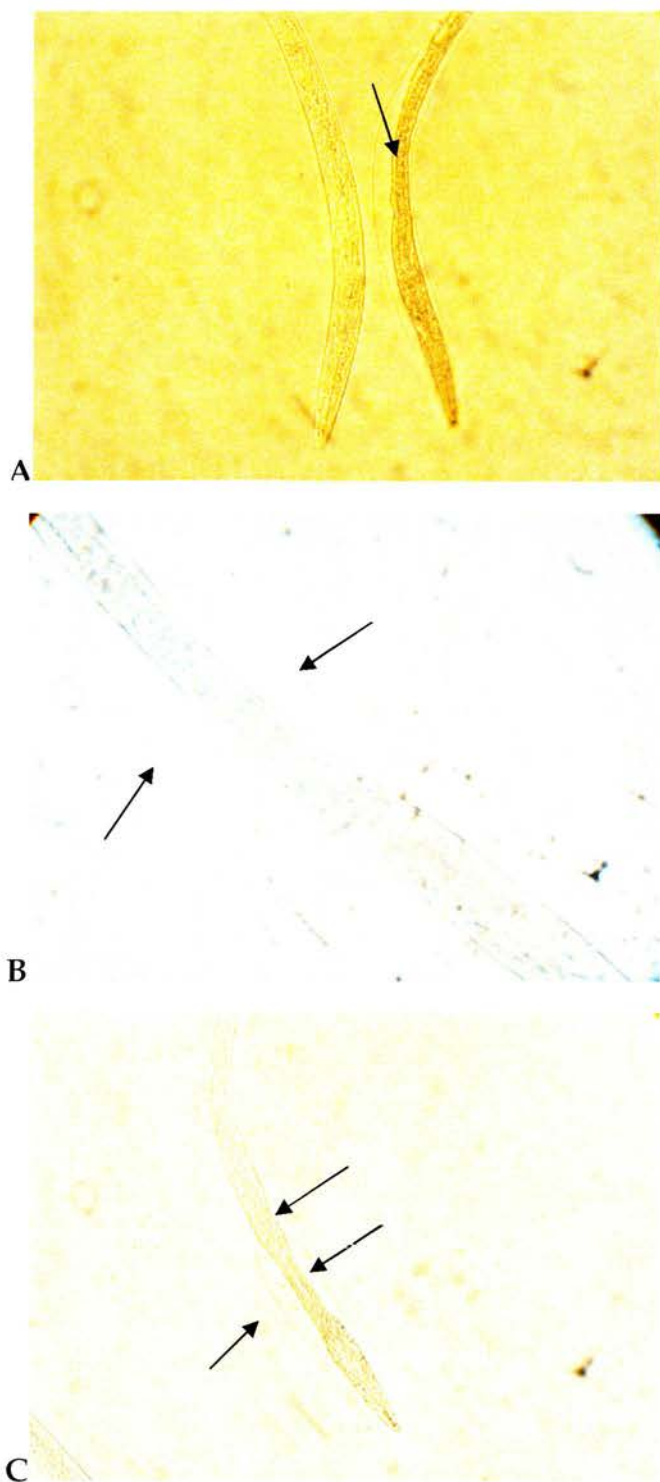


Figure 3.2. (A-C) Photographs of larvae from the larvae feeding inhibition assay (LFIA). (A) Shows a normal larva on the left hand side of the picture and indicated with an arrow the one with the outer tegument blistered (20x). (B and C) Shows blistered larvae at different magnifications (40x and 20x, respectively).

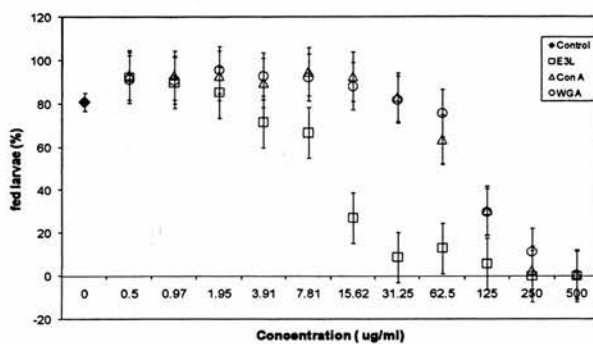
Table 3.3. IC50 values (means \pm s.e.) and range for L1 of three gastrointestinal parasites incubated in different plant lectins

Parasites	Lectins	IC50			
		Mean ($\mu\text{g/ml}$)	s.e.	Range	
<i>T. circumcincta</i>	E3L	7.3	1.2	5.1	9.4
	Con A	59.1	32.4	26.6	91.5
	WGA	78.9	11.2	62.8	100.3
<i>H. contortus</i>	E3L	8.3	1.4	6.1	11.0
	Con A	58.7	11.9	43.0	81.9
	WGA	69.4	8.1	61.0	85.5
<i>T. colubriformis</i>	E3L	4.3	1.7	0.9	6.3
	Con A	8.1	7.0	1.1	15.0
	WGA	28.0	14.1	10.7	56.0

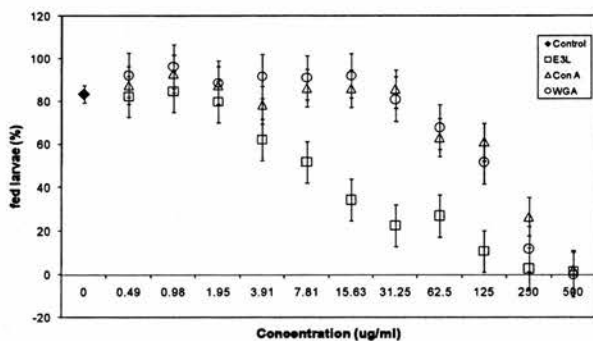
Parasites $P = 0.07$; Lectins $P = 0.0006$.

The % of fed larvae tested at different concentrations ($\mu\text{g/ml}$) is shown in Figure 3.3. E3L was clearly the lectin that was most effective at lower concentrations. In addition, *T. colubriformis* tended to have a higher susceptibility. For all experiments, using plant lectins and tropical plant extracts, the variations in feeding were expressed using standard error bars, including controls.

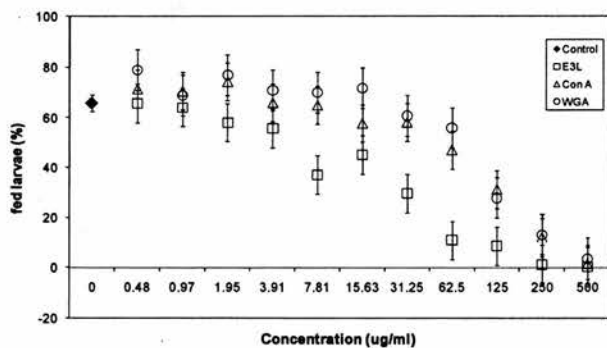
The effects that FET, Glu/Man and GlcNAc have in the *H. contortus* larval feeding assays are shown in Figure 3.4 (A, B and C). Larval feeding was reduced in FET, Glu/Man and GlcNAc compared with unexposed controls. There was little difference in effect at concentrations of FET ranging between 0.25 and 1.5 mg/ml, on average 63% of the larvae was able to feed. However, larval feeding was reduced to 16% at 3.0 mgs/ml FET. For the other 2 inhibitors there was no evidence of a dose dependant effect on feeding behaviour which was maintained at an average value of 51% for Glu/Man and 63% for GlcNAc.



A. *H. contortus*

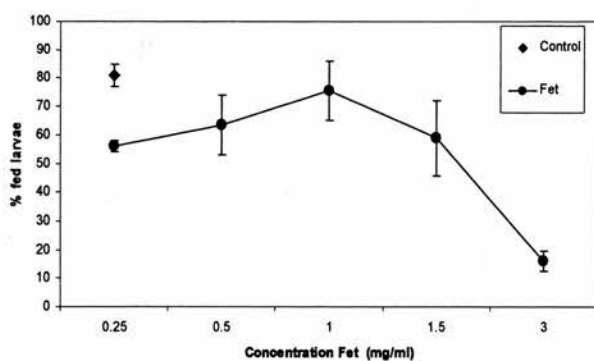


B. *T. circumcincta*

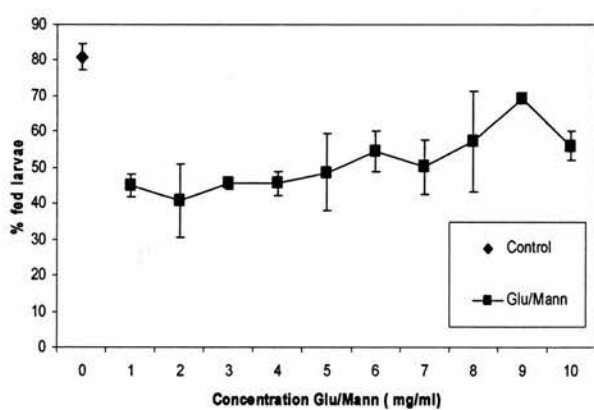


C. *T. colubriformis*

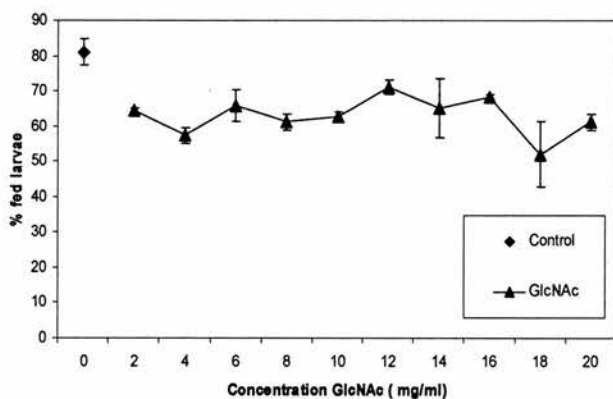
Figure 3.3. Effect of plant lectins on the feeding of first stage larvae of the three most common gastrointestinal parasites of sheep. **A.** *H. contortus*, **B.** *T. circumcincta* and **C.** *T. colubriformis* (means \pm s.e.).



A. Effect on the feeding behaviour of the larvae of FET, inhibitor of E3L.



B. Effect on the feeding of the larvae of Glu/Man, inhibitor of Con A.



C. Effect on the feeding of the larvae of GlcNAc, inhibitor of WGA.

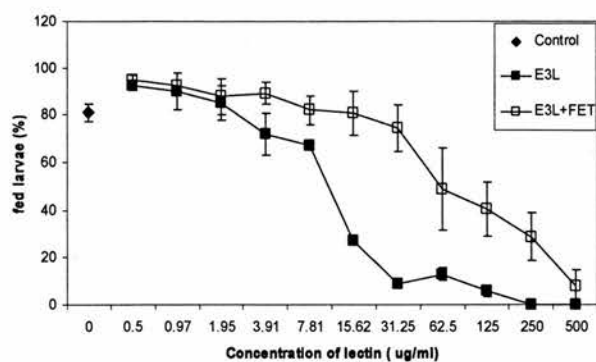
Figure 3.4. Effect of the plant lectin inhibitors FET, Glu/Man and GlcNAc on the feeding of first stage larvae of *H. contortus* (means \pm s.e.).

The efficiency of the lectin inhibitory carbohydrates blocking lectin activity is shown in Figure 3.5. Addition of FET to the E3L considerably reduced the effects of the lectin at all but the highest concentration of lectin. This indicated that the anti-feeding property of the lectin was dependent on its carbohydrate binding ability (Figure 3.5 A). In contrast, the addition of Glu/Man or GlcNAc had little effect on feeding by larvae treated with Con A or WGA respectively (Figure 3.5 B and C). The anti-larval properties may be a result of interactions between compounds in addition to binding of carbohydrates by the lectins.

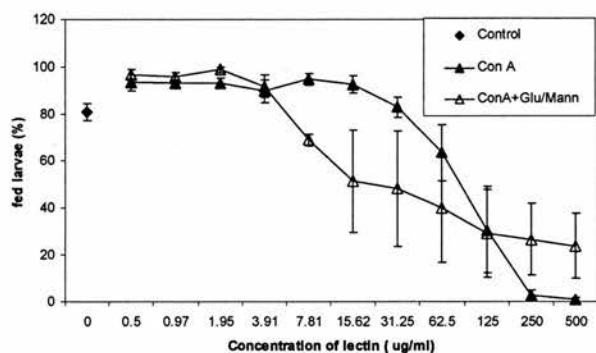
3.3.2. Binding sites of the lectins to the larvae

The results of the binding assays are shown in Figures 3.6. and 3.7. Figure 3.6. shows the control larvae incubated in water showing the fluorescent green bacteria (FITC labelled *E. coli*) within the oesophagus/intestinal cell region after feeding. As Figure 3.6 B shows the binding of biotinylated WGA appeared to be confined to the surface cuticle of the larvae.

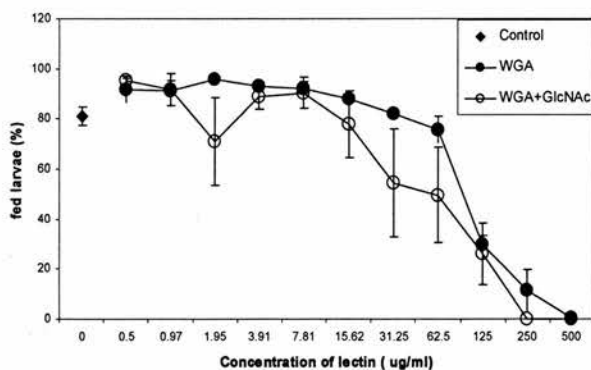
The immunohistochemistry of the worm sections are shown in Figure 3.7. Following incubation of sections with FITC labelled Con A fluorescent staining was observed throughout the organ structures as well as on the cuticle. Weak fluorescence was also observed in the control (non FITC-Con A treated) sections.



A

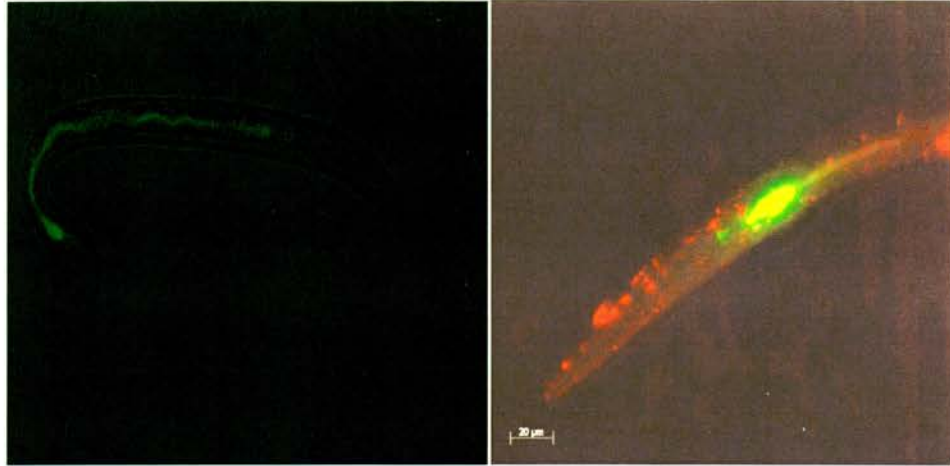


B



C

Figure 3.5. Effect of plant lectins and their inhibitors (in a fixed amount shown in brackets) on the feeding of first stage larvae of *H. contortus* (means \pm s.e.). **A.** E3L and E3L+ FET (300 μ g/ml). **B.** Con A and Con A+ Glu/Man (5 mg/ml of each). **C.** WGA and WGA+ GlcNAc (13 mg/ml).



A.

B.

Figure 3.6. Pictures of *T. circumcincta* L1 larvae from LFIA. **A.** Fed larvae (40x) showing the FITC-*E. coli* in the oesophagus/intestinal cell region. **B.** Fed larvae (40x) also showing the FITC-*E. coli* in the oesophagus/intestinal cell region and incubated in Biotin WGA+SAAF (streptavidin-af). Shows the red binding in the surface (biotin WGA was used in a concentration of 500 $\mu\text{g/ml}$).

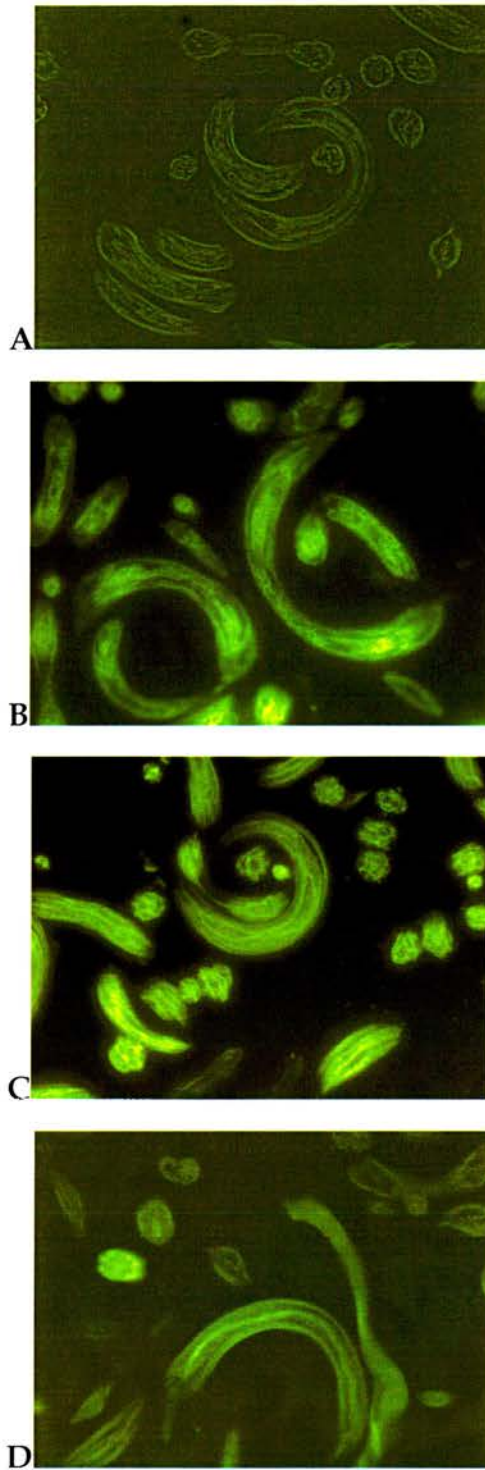


Figure 3.7. Pictures (40x) of worm sections of *H. contortus* L3. **A.** Control picture of worm section with no FITC labelled lectin. **B.** FITC labelled Con A. **C.** FITC labelled WGA. **D.** FITC labelled PHA.

3.3.3. Experiment 2: LEA using plant lectins

The effect of plant lectins on larval exsheathment is shown in Figure 3.8. None of the lectins used at 1000 $\mu\text{g/ml}$ for one hour had any effect on the exsheathment of L3 of *T. circumcincta*. For this reason, no other concentrations or parasite species were studied with this assay.

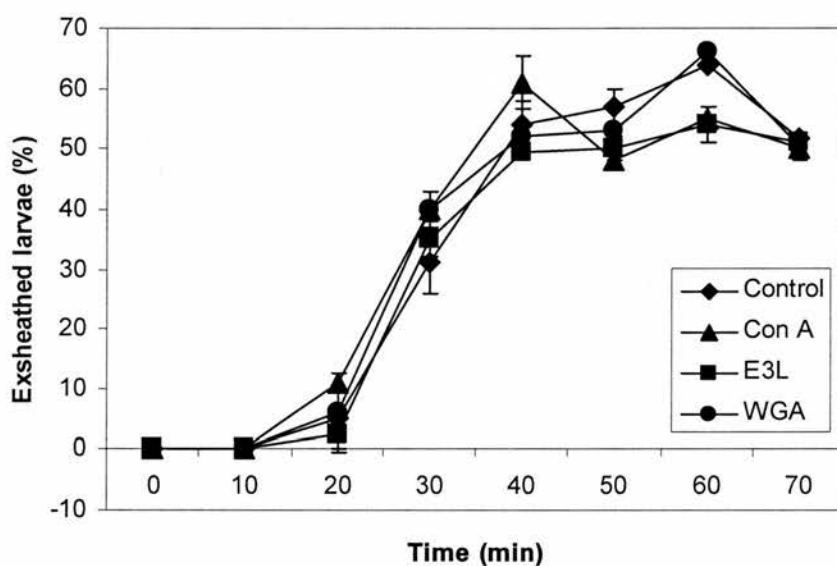


Figure 3.8. Effect of plant lectins on the exsheathment of *T. circumcincta* L3. The larvae were incubated for 3 hours in PBS (control) or in plant lectin solutions (Con A, E3L or WGA) at a concentration of 1000 $\mu\text{g/ml}$ (means \pm s.e.). (♦) PBS control, (▲) Con A, (■) E3L and (●) WGA

3.3.4. Experiment 3: LFIA using tropical plant aqueous extracts

Figure 3.9 shows the effect of the aqueous plant extracts administered at concentrations ranging from 0.2 to 10 mg of plant extract/ml on the feeding behaviour of *H. contortus* 1st stage larvae. GLI and LEU had the higher inhibitory effect. Because of the potent effect of GLI, this was re-tested at a range of lower concentrations (0.005-10 mg/ml). On the other hand NEE, NAC and MOR had a weaker effect on the feeding of the larvae.

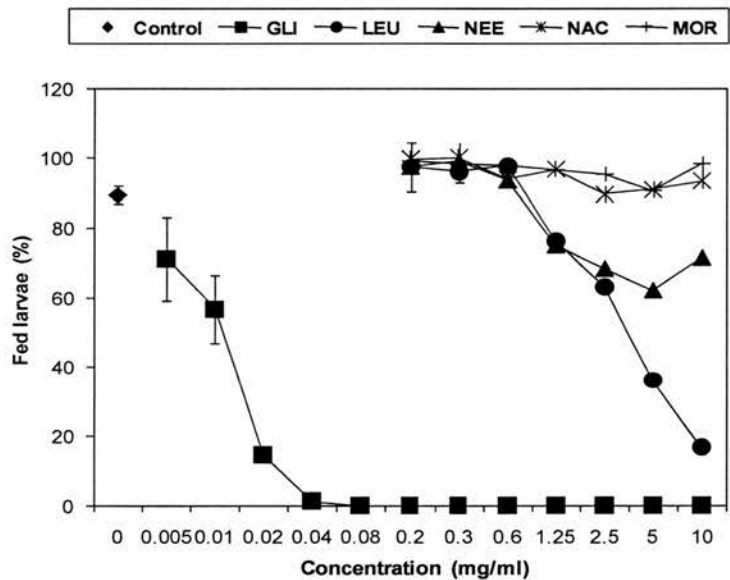


Figure 3.9. Effect of *Gliricidia sepium* (GLI), *Leucaena leucocephala* (LEU), *Azadiractha indica* (NEE), *Trichanthera gigantea* (NAC) and *Morus alba* (MOR) on the feeding of first stage larvae of *H. contortus* (means \pm s.e.).

Table 3.4 shows the concentrations of GLI and LEU needed to inhibit the feeding of 50% of the larvae exposed to these plant extracts. The concentration needed for inhibiting 50% of the larvae was less in GLI than in LEU.

Figures 3.10 and 3.11 show the effect of FET or PEG when used in combination with the plant extracts. When FET and PEG were tested independently with the GLI extract the effect on feeding inhibition was variable depending on the concentration of the extract. At higher concentrations of GLI, both PEG and FET had little effect, whereas at the lower concentration of 0.039 µg/ml of GLI, 38% and 20% of the larvae were able to feed in the presence of FET and PEG, respectively, whereas in their absence only around 1% of larvae were able to feed. For LEU, addition of FET and PEG had a more pronounced effect and a higher proportion of LEU-treated larvae were able to feed when FET or PEG was added to the medium. The findings indicate that larval food intake inhibition caused by GLI and LEU was a result of the action of multiple factors of which lectins may be one component.

Table 3.4. IC50 values (means ± s.e.) and range for L1 *H. contortus* L1 larvae incubated in *Gliricidia sepium* (GLI) and *Leucaena leucocephala* (LEU) plant extracts (*P*<0.001).

Plant species	IC50 values			
	Mean (mg/ml)	s.e.	Range	
<i>Gliricidia sepium</i> (GLI)	0.015	0.001	0.012	0.017
<i>Leucaena leucocephala</i> (LEU)	3.465	0.144	3.192	3.759

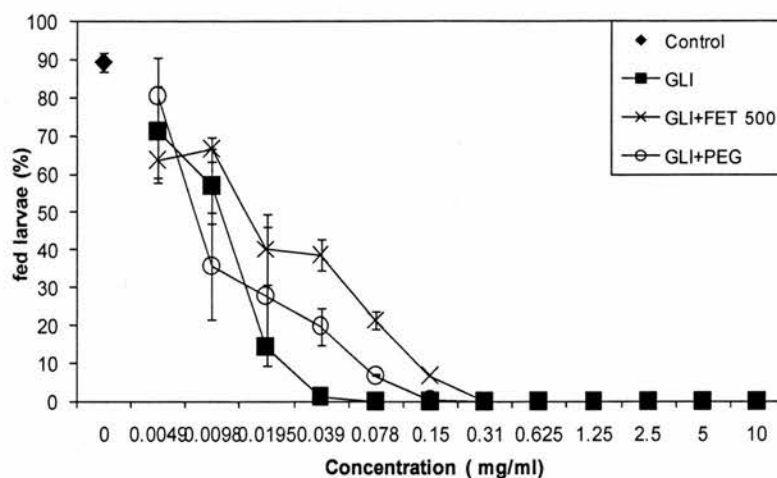


Figure 3.10. Effect of *Gliricidia sepium* (GLI), *Gliricidia sepium* + fetuin (GLI+FET) and *Gliricidia sepium* + polyethylene glycol (GLI+PEG) on the feeding of first stage larvae of *H. contortus* (means \pm s.e.)

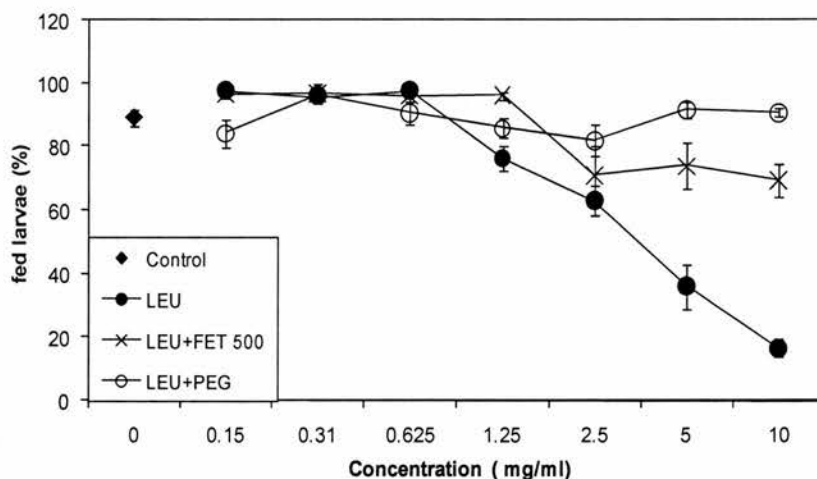


Figure 3.11. Effect of *Leucaena leucocephala* (LEU), *Leucaena leucocephala* + fetuin (LEU+FET) and *Leucaena leucocephala* + polyethylene glycol (LEU+PEG) on the feeding of first stage larvae of *H. contortus* (means \pm s.e.).

3.4. DISCUSSION

3.4.1. Experiment 1: LFIA using plant lectins

The present studies have demonstrated for the first time that plant lectins, in particular PHA, can have a direct inhibitory effect on the feeding behaviour of larvae of ovine GI nematodes *in vitro*. Moreover they also provide some evidence suggesting that lectins may contribute to the anthelmintic effects of some tropical forage plant extracts, such as *G. sepium* and *L. leucocephala*, since inhibition of larval feeding could be partly abrogated by addition of FET to medium. However, these are preliminary findings and further studies will be required to more accurately determine their impact.

A clear dose-response was found for the three plant lectins tested. PHA-E3L was significantly more effective an inhibitor of larval feeding behaviour than Con A or WGA. Within the three parasite species investigated, *T. colubriformis* tended to be the most sensitive GIN to the action of the plant lectins although this effect was not quite significant. The effects of PHA-E3L were clearly dependent on the carbohydrate-recognition / binding capacity of the lectin because they were inhibitable by FET. The modes of action of Con A or WGA was less clear and may involve both carbohydrate recognition / binding and other unrelated interactions of the lectin protein with the host. No effect of plant lectins was found on the process of artificial exsheathment of larvae. Additionally, studies in which labelled lectins were applied to nematodes suggest that the lectins can bind to all the exposed surfaces of the

larvae and this might affect their free mobility, although a more direct cytotoxic activity cannot be excluded.

These findings that extracts of forage crops and seeds have significant anthelmintic activity are in agreement with Gordon *et al.* (2007) who tested more than 500 plant extracts using this bioassay with *T. circumcincta*. The results showed that around 23% of their plant extracts were bioactive having IC₅₀ values of less than 1.25 mg/ml. They used LFIA findings to characterise these plants and select candidates for secondary *in vitro* screening using different assays with other parasite species. Both their findings and the ones in this chapter support the view that the LFIA can be a useful tool for screening for anthelmintic activity in PSM rich plants. In the Gordon study the top ten plant extracts had LFIA values ranging from 0.015 to 0.065 mg/ml, a similar range to that seen with PHA-E3L (0.006), Con A 0.042 and WGA 0.059 in the present studies.

Few studies have evaluated the potential of plant lectins as anthelmintics. However, Tobata-Kudo *et al.* (2005) tested the effect of various plant lectins, such as Con A, WGA and SBA on the chemokinesis of the infective third-stage larvae (L3s) of *Strongyloides ratti* *in vitro*. The binding sites of FITC Con A to the larvae were also studied. They found that the plant lectins at a concentration of 0.5 mg/ml affected the migration pattern of *S. ratti* L3s because they bound to chemosensors of amphids or the inner labial sensilla of the nematode. This may suggest that, in binding to these important sensory organs, lectins could be affecting neuromuscular activity and thus could disrupt locomotor behaviour.

Other studies have investigated the binding of plant lectins to different parasite species. Rao *et al.* (1987, 1989) showed that WGA binds to the surface of infective larvae of *Wuchereria bancrofti* but not to *Brugia sp.* suggesting that lectin-specific binding may be used as a diagnostic technique to differentiate these two species. Colditz *et al.* (2002) showed that *H. contortus* eggs stained strongly with peanut agglutinin (PNA), Con A, *Ricinus communis* agglutinin (RCA) and *Maclura pomifera* lectin (MPA) but *Trichostrongylus* spp. stained positively only with MPA. No reaction with PNA, Con A or RCA was evident with *Trichostrongylus* spp. Lectins have potential as tools to aid in identification or differentiation of species involved in natural GIN infections.

In the present study, preliminary investigations on the possible mode of action and target of the lectins *in vitro* provided some interesting findings. Whilst the results were inconclusive, it was noted that WGA induced visible damage to the cuticle of the parasite similar to that seen with plant extracts rich in cysteine proteases (Steppek *et al.*, 2005, 2006, 2007a, 2007b, 2007c). The cause of this pathology remains unknown but would be worthy of further investigation using protease inhibitors and more detailed pathological investigation using scanning electron microscopy.

In summary the LFIA screening study has identified three new PSM: the lectins PHA-E3L, Con A and WGA that strongly inhibit larval feeding *in vitro*. The actions of PHA-E3L were inhibitable by FET and thus clearly dependent on the carbohydrate recognition / binding properties of the lectin. In contrast, the actions of Con A and WGA could not be prevented by their inhibitory sugars. This suggests their anti-larval properties may be a result of multiple interactions with the larvae of which binding to specific carbohydrates is only one. Nonetheless, all three lectins have potential for

use in control of nematode infections and could be studied in more depth using *in vivo* studies.

3.5.1. Experiment 2: LEA using plant lectins

PHA-E3L, Con A and WGA had no significant effect on the exsheathment process of L3 larvae of *T. circumcincta*, even at lectin concentration of 1000 µg/ml. This is contrary to findings with tannins that have been shown to delay or inhibit the exsheathment process of nematodes (Bahuaud *et al.*, 2006). In that study exsheathment of *H. contortus* and *T. colubriformis* were studied after incubating L3s in the extract of tanniferous plants: heather (*Erica erigena*), pine tree (*Pinus sylvestris*), and chestnut tree (*Castanea sativa*). There was significant delay in the exsheathment of both species with pine tree extract and a significant delay of *H. contortus* exsheathment after treatment with heather extract but no effect on *T. colubriformis*. Chestnut tree extract greatly inhibited exsheathment of both species but unlike the effects of the heather and pine tree extract, the action of chestnut tree extract could not be reversed using PEG, generally described as a tannin inhibitor.

Lectins have been shown to interfere with the larvae feeding. However, their ability to interfere with larvae may be stage-dependent. Thus, larvae that have developed normally to L3 do not appear to be affected by exposure to lectin and are capable of normal exsheathment.

3.5.2. Experiment 3: LFIA using tropical plant aqueous extracts

This study demonstrates that the plants chosen as controls, MOR and NAC, had no anthelmintic effect *in vitro*. However, Gordon *et al.* (2007) established an arbitrary threshold for screening aqueous plant extracts for anti-parasite activity. Plants with IC₅₀ values below a threshold concentration of 1.25 mg/ml were considered as having anthelmintic activity. Using this threshold, GLI which has an IC₅₀ value of 0.015 mg/ml appears to have potential. This is not unexpected because this plant is widely recognised in tropical countries as a medicinal plant (Cáceres *et al.*, 1991, 1993) with trypanocidal activity (Berger *et al.*, 1998). In the case of trypanosomes, Berger and colleagues showed that the ethanol extract of *G. sepium* bark had the highest IC₉₀ against trypomastigotes a developmental stage of Trypanosomatidae living mostly free in the blood of vertebrate hosts) for this plant with the value of 381.5 mg/ml.

LEU extracts had potent anthelmintic properties. However, according to the arbitrary criteria of Gordon *et al.* (2007), its IC₅₀ 3.1 mg/ml would not be considered anthelmintic. Extracts of *L. leucocephala* may however vary in their potency since Alonso-Díaz *et al.* (2008a, 2008b) found that a *L. leucocephala* extract affected both the migration and exsheathment of L3s of *H. contortus* and *T. colubriformis*, at a concentration of 1.2 mg of extract/ml. *L. leucocephala* extract inhibited the migration of *H. contortus* by 44% relative to the control larvae in PBS and the effect was reversed using polyvinyl polypyrrolidone (PVPP) a tannin inhibitor. Exsheathment of L3s of *H. contortus* was reduced by *L. leucocephala* extract (only 10.64% of the larvae

were exsheathed after 60 min) whereas the control larvae in PBS were totally exsheathed (100%).

Rojas *et al.* (2006) tested the effect of *G. sepium* and *L. leucocephala* on *H. contortus* burdens in gerbils and lambs, using forages that contained 8.1 and 129.5 g CT/kg DM, respectively. Both species had anthelmintic effects on lambs and gerbils, greatly reducing the numbers of L4s and L5s detected (70% less than in non-treated infected controls).

Another explanation for the differences in activity seen between studies could simply be that PSM in addition to CT may be contributing to the anthelmintic effects. In the present study FET was more potent than PEG in inhibiting the anti-larval effects of GLI extract. This suggests that in GLI the anthelmintic activity, could, at least in part, be due to lectins in the extract. However, D'Mello (1992) described *G. sepium* and *L. leucocephala* as having no lectins. In contrast, Grant *et al.* (1995) found low levels of lectin in *L. leucocephala* seeds.

NEE is a widely studied plant because of its medicinal and anthelmintic properties. However, in this study the results obtained with NEE were very poor compared with GLI and LEU. Results reported in the literature for *A. indica* can also be inconsistent; some positive anti-parasitic effects were found by Pietrosevoli *et al.* (1999) using *A. indica* leaves as supplements for grazing cattle in a tropical dry forest area of Venezuela. They found that the eggs per gram of fresh faeces (epg) were significantly reduced in cattle given *A. indica*. Chandrawathani *et al.* (2006) found that feeding fresh *A. indica* leaves to adult Santa Ines cross-breed sheep did not affect FEC compared to non-treated / infected controls (from day 12 onwards the *A. indica* group had between 1160

to 3733 epg *vs.* 2140 to 11117 epg in the control group). In addition, the worm burden estimates were significantly higher for the control group (3242) compared to the *A. indica*-treated one (1004). In contrast, others report that *A. indica* has no anthelmintic effect; Githiori *et al.* (2003) found no effect of this supplement on faecal egg count and total worm count in mice infected with *H. polygyrus*. Hördegen *et al.* (2003) found that *A. indica* reduced egg counts by only 5.2% and had no effect upon *H. contortus* and *T. colubriformis* worm burdens. These differences in efficacy could be due to factors related to the plant age, drying method, season of harvest, and the 'stress' that the plants have been exposed to.

These studies *in vitro* have provided novel data on the action of lectins against GIN, and have identified a new class of PSM, which could be useful for limiting GIN infections in the field, if they are found to maintain their efficacy *in vivo*. The aim of the next chapter will be to conduct an *in vivo* experiment in which parasitised sheep will be treated with a kidney bean lectin preparation.

EFFECT OF ORAL DOSING WITH SEMI-PURIFIED PHYTOHAEMAGGLUTININ LECTIN ON THE CONTROL OF GASTROINTESTINAL PARASITES AND THE GROWTH OF SHEEP

4.1. INTRODUCTION

In the previous chapter plant lectins studies *in vitro* using the LFIA described for the first time that these compounds can have a dramatic effect on the feeding of GIN first stage larvae, although no effect was found on the exsheathment process of L3 larvae.

The present chapter describes an *in vivo* study using semi-purified PHA lectins (extracted from *Phaseolus vulgaris*) on parasitised lambs experimentally trickle-infected with two GIN species, the abomasal nematode *T. circumcincta* and the intestinal nematode *T. colubriformis*.

PHA also called kidney bean lectin was chosen because of its potent inhibition of feeding of L1 described in Chapter 3. In addition, kidney beans are widely available, generally contain high (~20g/kg DM) amounts of lectin, and the lectins are relatively easy to extract in sufficient quantities in a semi-purified form. The stability of PHA lectin to proteolysis and its potential toxicity have been extensively studied in different species (Banwell *et al.*, 1984; Bardocz *et al.*, 1995, 1996; Carbonaro *et al.*, 2000; Fitches *et al.*, 2001; Kennedy and Nager, 2006) and were also taken into account when

considering which lectin to use. Dietary proteins are, generally speaking, rapidly degraded by digestive enzymes, during their passage through the gut. However, PHA can resist degradation by proteases *in vitro* (Carbonaro *et al.*, 1997) and also resists degradation during transport through the rat gastrointestinal tract *in vivo* (Pusztai, 1991), and up to 90% of the PHA ingested can be recovered intact in the intestine or faeces (Oliveira *et al.*, 1994). However, during passage of PHA through the intestine of rats it binds to sugar complexes present on the epithelium, interferes with cellular metabolism and causes pathological changes, in particular severe disruption of duodenal and jejunal enterocytes and the epithelial integrity (Pusztai *et al.*, 1979).

Intake of PHA (525 mg/kg BW/d) daily for up to 10 days caused hyperplasic growth of the small intestine with a drastic overgrowth of mannose-sensitive *E. coli*, in contrast to healthy and well fed rats where *E. coli* is present in low numbers because their small intestine has mature enterocytes with few mannosylated adhesion sites (Pusztai *et al.*, 1993). This growth of *E. coli* was dose-dependant, and bacterial counts rose from 10^2 - 10^3 /g tissue to 10^8 - 10^9 counts/g tissue when the PHA intake was in a range of 225-760 mg/kg BW/d. However, when the intakes were below 225 mg/kg BW/d, counts were similar to control ones (10^2 - 10^3 /g tissue) (Wilson *et al.*, 1980; Pusztai *et al.*, 1993; Grant, 1999). Furthermore, at intake of 225 mg /kg BW/d or less PHA-induced growth of the small intestine and modification of epithelial composition is very dose-dependent. The mechanisms that trigger this process are not totally known; PHA binds extensively to the epithelium, specifically to the absorptive cells, goblet cells and neuroendocrine cells (Pusztai, 1991). At intakes of 225 mg/kg BW/d or less PHA-induced changes in the gut are fully reversible after removal of lectin from the diet. Thus, from

these studies it can be demonstrated that the ingestion of PHA can have a profound effect on the pathology and physiology of the intestine. However, there have been few studies investigating the impact of PHA on GIN or indeed gut responses to infection.

Karmańska and Michalska (1978) found that injecting mice with 10 mg/kg body weight of PHA-P 24 hours before infection with *T. spiralis* caused an enhanced stimulation of MMC and increased stimulation of T-cells compared to control animals. In a further study Karmańska *et al.* (1996) injected mice 24 h before *Trichinella* infection with PHA-P. Post-infection they collected samples of spleen, mesenteric lymph node, jejunum and masseter muscles and found that PHA-treated mice had higher counts of CD4+ cells in intestine and muscles compared with controls and CD8+ cells were stimulated in the muscles. Piekarska *et al.* (2008) found PHA increased apoptosis amongst splenic lymphocytes and inflammatory muscular infiltrations and reduced apoptosis in mesenteric lymph nodes on day 7 post-infection with *Trichinella*. They also found apoptotic changes in intestinal mucosa cells following PHA-P treatment. These limited studies in rodents indicate that PHA can have profound immunomodulating effects, and may have the ability to enhance local and systemic immune responses against GIN.

In addition to the above studies with rodents, there are several investigations that involve the use of PHA in skin testing, to assess the immunocompetence of birds infested with ectoparasites. This involves the injection into the skin of low doses of PHA, which invokes a local proliferative T cell response which can be monitored by an increase in skin thickness, and further

demonstrates the immnostimulatory properties of PHA (Johnsen and Zuk, 1999; Kennedy and Nager, 2006; Owen and Clayton, 2007; Tella *et al.*, 2008).

The present study, therefore, investigated the use of the lectin PHA for the control of GIN in lambs. This control could be through the ability of the lectin to enhance local immune mechanisms, as discussed above, or through the direct 'anti-parasitic' effects demonstrated in Chapter 3, or a combination of these mechanisms since they are not mutually exclusive. Factors that were considered during the design of this study were the availability and dose of semi-purified PHA, frequency of dosing and the duration of the experiment. A dose of ~2 mg PHA/ kg BW/day was considered to be practically possible, and was well below the levels that have been shown to be toxic in laboratory animals. It was also considered that this dose should also supply transient concentrations of PHA similar, or above, to those that were shown to have a direct *in vitro* effect on the larvae. The experiment was undertaken over a period of 6 weeks, which was a compromise based on the available quantities of PHA, and the ability to identify and quantify any effector mechanism(s). Thus, any direct effect on the FEC would be detected by 6 weeks of worm challenge, and the analysis of abomasal and intestinal tissues at termination would provide evidence of enhanced quantitative or qualitative changes in the mucosal effector cell populations during the initial stages of infection when effective host immunoregulatory responses do not occur (Seaton *et al.*, 1989a, 1989b).

4.2.MATERIALS AND METHODS

4.2.1. Experimental design

Twenty-four weaned mixed breed lambs that were reared indoors under conditions that were designed to preclude accidental exposure to nematodes, were allocated to one of four groups (n=6) that were balanced for both sex (males and females) and initial liveweight (34.3 ± 4.2 kg) and randomly allocated to treatments in a 2 x 2 factorial design (Table 4.1). The first factor was parasite infection, with animals either infected (+P), or not (-P), with the equivalent of 1000 infective L3 *T. colubriformis* and 1000 infective L3 *T. circumcincta* larvae per day in a three-times weekly trickle-dosing regime. Larvae were suspended in water and were administered orally on Monday, Wednesday and Friday of each week for six weeks. The mixed infection with these species was chosen in order to be able to measure the impact of lectin administration in two different sites of the animals at the same time.

The second factor was lectin administration, with animals receiving either a daily dose of 200 mg of a semi-purified PHA extract or a sham treatment. The semi-purified PHA extract contained inhibitors and other potentially active compounds that can not be excluded when large amounts of lectins are needed for *in vivo* purposes. The extract was 40.24% PHA w/w, with 200mg of extract equivalent to 80 mg pure PHA; initial daily doses of PHA were 2.3mg/kg BW/d. Both PHA (+L) and water (sham; -L) treatment was administered orally five times weekly on each day from Monday to Friday. Semi purified PHA lectin for all animals (+L) was reconstituted each morning

for fresh dosing, using tap water, a cold plate with magnetic stirrer and a magnet. The exact dose of lectin was placed in individual syringes for each animal. All animals were monitored for any side effects of the lectin treatment.

Table 4.1. Description of the different factors and treatments and purpose of each treatment involved in the lectin *in vivo* study.

Group	n	Treatment	Frequency of treatments	Purpose
-P-L (Control)	6	No parasites (-P) no Lectin (-L)	N/A	Positive Control: normal values for growth rate of non-parasitised sheep, normal abomasal and intestinal tissues to compare the immune response of the animals and normal values for blood
-P+L	6	No parasites (-P) + Lectin treatment (+L)	Lectin (oral dose of 80 mg of PHA, 5 times/week)	To determine the effect of lectins on normal, non-parasitised animals
+P-L	6	Parasites (+P) + Lectin treatment (-L)	Parasite challenge (1000 <i>T. colubriformis</i> +1000 <i>T.circumcincta</i> /animal, 3 times/week throughout the experiment)	Control, parasite infection with no lectin
+P+L	6	Parasites (+P) + Lectin treatment (+L) (80 mg of semi purified PHA/animal per day)	Parasite challenge (described above) + Lectin (described above)	To demonstrate the effect of PHA on the parasite infection, through monitoring FEC, and comparing total worm burdens and local immune cells at termination

4.2.2. Animal feeding and sampling

Animals were group housed within their treatments with access to fresh water and were offered a diet of fresh hay *ad libitum* in addition to approximately 0.5 kg of concentrate per animal per day. Pooled weekly samples of the hay and concentrate offered were collected for chemical analyses which are given in Table 4.2. Liveweight was recorded weekly at the same time of day at each time point and also on the final day of the experiment prior to slaughter for the estimation of total liveweight gain.

Table 4.2. Chemical composition of a pooled sample of the feeds offered to the animals during the experiment.

	Grass Hay	Concentrate
Dry Matter (g/kg fresh matter)	929	937
Crude Protein (g/kg DM)	89	179
Metabolisable Energy (MJ/kg DM)	85	111
Fermentable Metabolisable Energy (MJ/kg DM)	81	98
Digestible Organic Matter (D-value, % DM)	59.3	69.2
Total Starch (g/kg DM)	23	186
Ash (g/kg DM)	44	89
Oil (g/kg DM)	11	36

4.2.2.1. Clinical findings

Lectin dosed animals were observed daily for possible external effects or symptoms due to this treatment. Because this is a pilot study and the correct dose with PHA has not been studied before, 80 mg/animal/day was chosen as a safe lectin administration. However, it was important to be sure that no diarrhoea or other adverse symptoms appeared.

4.2.2.2. Blood sampling and plasma analysis

Blood samples were collected weekly by jugular venepuncture into heparinised tubes (vacutainer systems, Becton Dickinson, UK), were centrifuged at 2500 rpm for 15 min and the plasma was removed and stored at -20°C until analysis for plasma total protein, albumin and inorganic phosphate performed using a microcentrifugal analyser (Ilab 300 Plus, Instrumentation laboratory, Warrington, Cheshire, UK). The kits used were: Total Protein (Instrumentation Laboratory, cat no. 18481300), Albumin (Instrumentation Laboratory, cat no. 18481800) and Phosphorus (Instrumentation Laboratory, cat no. 18481900). The standards were also from Instrumentation Laboratory, Serachem Control Level 1 (cat no. 18162412), Serachem Control Level 2 (cat no. 18162512). The calibrator was Referril G, (Instrumentation Laboratory, cat no. 18257000). All the analyses were performed according to the instructions from manufacturers.

4.2.2.3. Faecal sampling and analysis

Faecal samples were collected directly from the rectum twice weekly (Monday and Thursday) for the determination of the concentration of nematode eggs in the faeces (FEC) using the modified floatation technique (Christie and Jackson, 1982) and were expressed as epg. From week 4, a sub-sample of the faeces collected from infected animals was taken for coproculture, according to the methodology described by Kaufmann (1996). Briefly, from 3 to 20 g faeces/animal was incubated separately for 10 days at 22-23 °C. Following incubation, faeces were left to soak in water for 24 h to allow the larvae to exit the faecal mass before cleaning by baermannisation. The larval yield was calculated according to the number of larvae present after baermannisation as a proportion of the number of eggs in the faecal mass. The variables analysed were estimation of total number of recovered larvae from the faeces. From each coproculture a sub-sample of L3 was taken, exsheathed and speciated (MAFF, 1986) for the estimation of the proportion of *T. colubriformis* and *T. circumcincta* per treatment.

4.2.2.4. Slaughter and worm recovery

All animals were slaughtered on day 42 by stunning with a captive bolt followed by exsanguination through severance of the carotid artery and jugular vein. Immediately following slaughter the abomasum and first five metres of the proximal small intestine from all infected animals were excised. Worm recovery from the abomasum and small intestine was performed following incubation in physiological saline at 37°C for 4 hours as described by Jackson *et al.* (1984). Worm enumeration according to stage of development and sex was performed from a 1% aliquot and multiplied by

100 to give total worm numbers. Estimations of the total worm establishment were calculated using the total numbers of infective larvae given to each animal throughout the experiment compared with the number of worms recovered at slaughter $[(\text{Total worms recovered} / \text{Total worms given}) \times 100]$. Finally the female worm fecundity (or per capita fecundity) was estimated based on the numbers of females recovered at slaughter and the last FEC per animal just before slaughter.

4.2.2.5. *In vitro* direct challenge (IVDC)

The ability of abomasal tissue to prevent larval establishment was examined using the IVDC technique as described by Jackson *et al.* (2004). Briefly, fundic folds were excised from the abomasa that were retrieved, as previously described, and rinsed gently in warm physiological saline (0.85%) to remove the majority of adherent digesta. Three tissue samples measuring approximately 2 cm x 2 cm were removed from each animal. Each tissue section was placed into a well of a Corning six-well plate. Warm Hank's medium (Sigma Chemical Company, St. Louis, MI, USA) with 20 mmol l⁻¹ Hepes (Boehringer Mannheim GmbH, Germany) and 2 ml phenol red was added to 1 l of sterile H₂O and brought to pH of 7.6 with NaOH, was added to surround but not submerge the tissues. The barrels of 10ml syringes with the needle end removed were placed onto the centre of each piece of dissected tissue to provide an isolation cylinder to contain the larvae. Doses of approximately 2000 exsheathed *T. circumcincta* larvae were introduced in 0.5 ml of saline onto the mucosal surface of each tissue section within the isolation cylinder with sufficient pressure applied to the lid of the six-well plate to ensure an effective seal between the cylinder and tissue. The plates were transferred to an incubator (Stuart Scientific S160) and maintained at

38°C in a high oxygen concentration, which was achieved by gassing with pure oxygen for one minute. The time taken from slaughter to incubation was no more than 20 min. After incubation for three hours, the tissue samples were removed from the incubator and washed by repeated plunging for 30 times in warm physiological saline (wash). The tissue sections were then digested through incubation in pepsin and HCl for 12h (digest). Aliquots of wash and digest samples were taken and the number of larvae in each enumerated. The close association between larvae and the abomasal tissue was calculated as a percentage proportion of the total larvae recovered (wash +digest), that were found in the digest fraction

4.2.2.6. Histochemical and immunohistochemical analysis

Samples of abomasal and intestinal tissues from non-parasitised and parasitised animals were removed immediately following slaughter and fixed in either PBS containing 4% PF for 6 hours at room temperature (Newlands *et al.*, 1984) or in Zn salts fixative at room temperature for 24 hours (González *et al.*, 2001). After fixation, tissues were processed and embedded in paraffin; 5 µm sections were cut, mounted on slides and dried for 12 h at 40 °C. For PF fixed sections, general histochemical characteristics were assessed following haematoxylin-eosin staining. MMC and eosinophils were enumerated following staining with toluidine blue (Enerback, 1966) and carbol-chromotrope (Lendrum, 1944), respectively. In abomasum mucin-producing cells and in the intestine, goblet cells in the epithelium and paneth cells in the crypts were detected using Periodic Acid Schiffs (PAS) (Mantle and Allen, 1978). Neutral and acidic mucins were identified through alcian blue-PAS staining (Newlands *et al.*, 1990). Unfortunately, the Ab-PAS staining was not able to distinguish between the different types of mucin-

producing cells, and almost all goblet cells appeared to be stained homogeneously with PAS indicating the presence of neutral mucin. Stained cells were enumerated using a x10 eye piece containing a calibrated graticule and a x40 objective lens. Zn salts fixed tissue sections were employed for immunostaining. Pan T cells were detected with anti-CD3 (AntiCD3, Dako, Ltd, Ely, UK) at a dilution of 1/200. T helper cells were detected using antibody 17D Ovine CD4, isotype IgG1, Batch 160995 and type ascites at a dilution of 1/1000 (Hein *et al.*, 1987). Controls consisted of tissue sections where the primary antibody was omitted and instead the mouse serum (1/200) from IgG1 myeloma, clone MOPC21, purified immunoglobulin was used (Sigma catalog no. 026K4804). Stained cells were enumerated using the software ImageJ (1.41.g Wayne Rasband National Institutes of Health, USA), which contained a calibrated graticule. All cell counts from both PF and Zn salts fixed tissue sections were made systematically in the epithelium and mucosa from the mean of 10 graticule fields, and were expressed as cells/mm² of intestinal tissue.

4.2.3. Statistical Analysis

Analyses were performed using GenStat statistical software version 7.2 (Lawes Agricultural Trust, 2004) as a 2x2 factorial design with diet and infection as the factors unless otherwise stated. Prior to statistical analysis, FEC and worm burdens were log-transformed ($\log_{10}(x+1)$). All transformed data are presented as back-transformed means unless otherwise stated. Feed intake, liveweight and serum data were analyzed by REML with time included as a factor. Worm burdens, liveweight gain and intestinal tissue cell concentrations were analysed by using a general ANOVA, arithmetic means

with standard error are presented. For FEC, worm burden and coprocultures analysis was performed on data from infected animals; consequently infection was removed as a factor.

4.3. RESULTS

4.3.1. Clinical findings

No adverse reactions or other clinical problems were observed in animals dosed with 80 mg PHA/animal/d five days per week for 6 weeks.

4.3.2. Liveweight

Mean liveweight at day 0 was similar for all groups, being 34.3 ± 0.87 kg. Mean liveweights at day 42 were 40.75 ± 5.1 , 39.42 ± 2.7 , 36.3 ± 4.6 and 36.8 ± 4.7 kg for -P-L, -P+L, +P-L and +P+L, respectively. Overall, liveweights increased with time ($P=0.012$) and were reduced by infection ($P<0.001$) but were not affected by lectin treatment ($P=0.480$) (Figure 4.1).

4.3.3. Faecal egg counts and dry matter of faeces

Mean log₁₀ back-transformed FEC are shown in Figure 4.2. Overall, for samples taken at all times, there was an effect of time ($P<0.001$) but no effect of lectin treatment ($P=0.503$). For all faecal samples taken after day 22, there was an effect of time ($P=0.002$) and there tended to be an effect of lectin treatment ($P=0.070$). For samples taken between days 25 and 36, there was an overall effect of lectin ($P=0.033$) but not time ($P=0.294$). The FEC of all infected animal increased throughout the duration of the study with the exception of a temporary decrease in +P-L animals on day 39 only. Although

samples from the +P-L animals were checked twice, the FEC data were re-analysed with the aberrant observations on day 39 excluded. Mean log10 back-transformed FEC with data from all animals on day 39 excluded are given in Figure 4.3. Overall, for the re-analysed data for samples taken from day 25, FEC increased with time ($P=0.047$) and were greater in the +P-L group compared to the +P+L group ($P=0.002$).

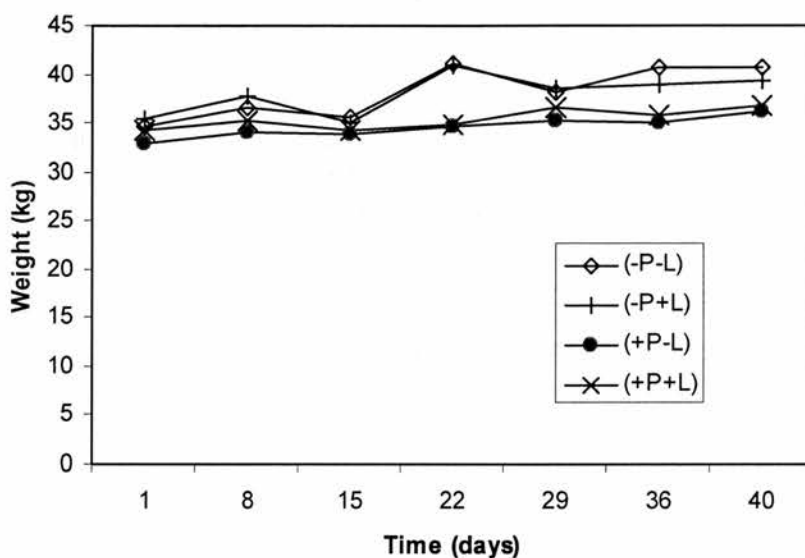


Figure 4.1. Liveweights of the animals throughout the experiment. The treatments result from the combination of factors P and L, where -P: non-parasitised animals, +P: parasitised animals, -L: no lectin treatment and +L: lectin treatment.

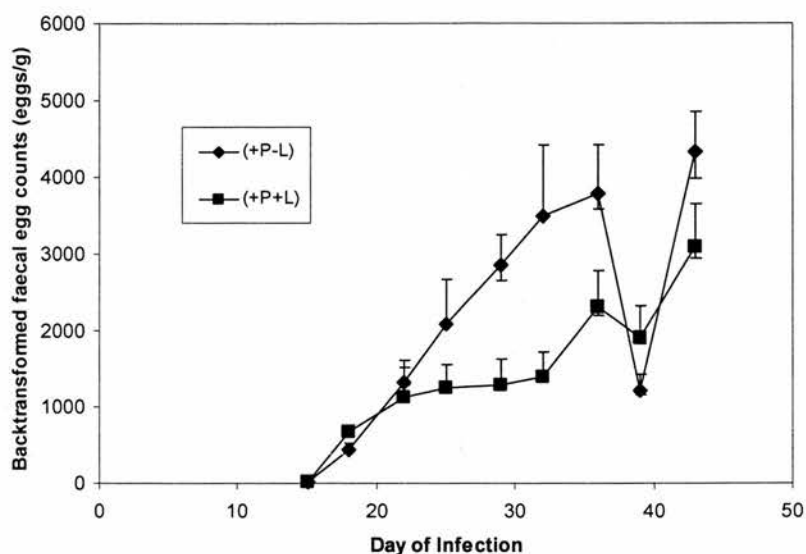


Figure 4.2. Mean backtransformed faecal egg counts (FEC; eggs per g) for parasitised animals that either were dosed with lectin (+P+L) or were not dosed (+P-L). Error bars represent 95% confidence intervals.

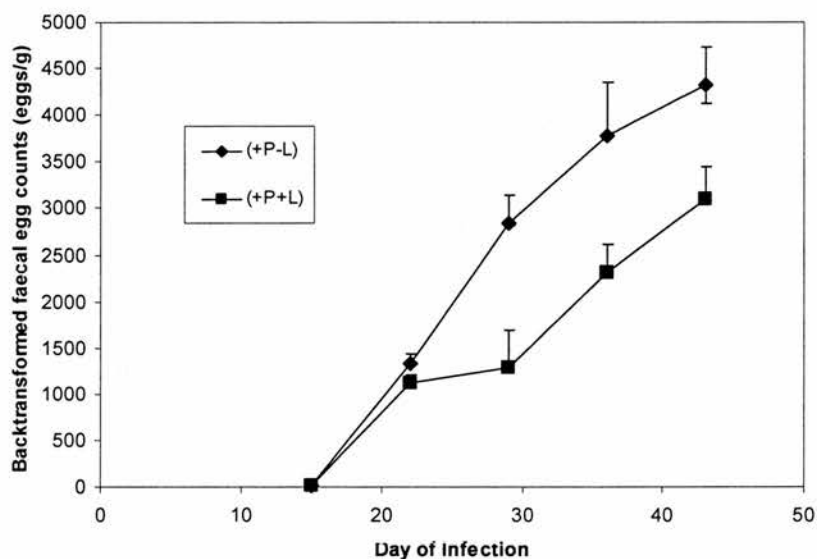


Figure 4.3. Mean backtransformed faecal egg counts (FEC; eggs per g) for parasitised animals that either were dosed with lectin (+P+L) or were not dosed (+P-L) with data from day 39 excluded. Error bars represent 95% confidence intervals.

Although some animals presented isolated cases of diarrhoea, treatments did not produce any scours that affected the dry matter, these were similar ($P=0.12$) between treatments with means \pm s.e. of 425.8 ± 30.3 , 414.4 ± 40.0 , 326.9 ± 24.3 and 403.1 ± 26.7 g/kg for -P-L, -P+L, +P-L and +P+L, respectively.

4.3.4. Coprocultures and species prevalence

There was also no difference between the percentage of each species recovered in the faecal cultures from animals that were either exposed or not exposed to lectin. The average percentage of *T. circumcincta* and *T. colubriformis* recovered from the control (+P-L) and the lectin treated (+P+L) groups was 39.3 ± 4.9 and 42.4 ± 5.1 (*T. circumcincta*) and 60.7 ± 4.9 and 57.6 ± 5.1 (*T. colubriformis*).

The number of larvae recovered from the coprocultures of the control and treated animals were not significantly different ($P=0.147$), with mean recoveries \pm s.e. of 309.3 ± 57.2 and 435.9 ± 63.8 L3s/g faeces for +P-L and +P+L, respectively.

4.3.5. Worm burdens

There were no significant differences in the total *T. circumcincta* and *T. colubriformis* burdens nor in the sex ratio or immature worm *vs.* mature worm numbers as a consequence of lectin treatment. The mean total burdens (\pm s.e.) for *T. circumcincta* and *T. colubriformis* in the control (+P-L) and lectin treated (+P+L) groups were 4416 ± 878 and 3475 ± 792 and 6708 ± 414 and 6500 ± 295.5 , respectively. Although more than 5% fewer *Teladorsagia* were

recovered from the lectin treated animals this difference was not significant. The overall establishment figures (the percentage of the total challenge recovered at post mortem) for the control animals (+P-L) and treated animals (+P+L) were 24.5 ± 4.9 and $19.3 \pm 4.4\%$ (*T. circumcincta*) and 37.3 ± 2.3 and 36.1 ± 1.6 (*T. colubriformis*) respectively. The *per capita* fecundity measured as total egg production per day per adult female worm was not significantly affected by lectin treatment with means of 136.8 ± 18.5 and 168.6 ± 34.1 for animals in the control (+P-L) and treated (+P+L) groups.

4.3.6. *In vitro* direct challenge (IVDC)

The % of larvae found to be closely associated with the abomasal tissue estimated as (100-larvae rejected) are shown in Table 4.3. Figures were estimated based on the individual factors (-P and +P, -L and +L) and their different combinations in treatments (-P-L, -P+L, +P-L and +P+L). Overall, larval association with the tissue was reduced by infection ($P < 0.001$) and tended to be reduced by lectin administration ($P = 0.063$).

Table 4.3. Percentages of larvae association to the abomasal tissue estimated from the *in vitro* direct challenge assay (IVDC).

Treatments	Larvae association (%)
-P-L	68.0
-P+L	57.8
+P-L	19.3
+P+L	5.3

Infection: $P < 0.001$ (s.e. 6.4); Lectin: $P = 0.063$ (s.e. 6.4); Interaction: $P = 0.780$ (s.e. 9.4)

4.3.7. Histochemical and immunohistochemical analysis

Histochemical and immunohistochemical cell counts for eosinophils observed in abomasal and intestinal tissues collected at slaughter are shown in Table 4.4. Overall, eosinophils concentrations in abomasal tissue sections displayed a lectin x parasite interaction ($P<0.001$) (Table 4.4.) which reflected an increase in parasitised animals that was increased also by lectin administration. For small intestinal tissue sections, eosinophils concentrations were increased by both lectin administration and by infection ($P=0.001$ for both). In addition, there was a tendency for an interaction between lectin and parasite infection ($P=0.087$) in which parasite infection tended to cause a larger increase in lectin treated animals compared to non-lectin treated animals.

Mean MMC concentrations for abomasum and small intestine are given in Table 4.5. Overall, there was no effect of either lectin administration or parasite infection on the numbers of MMC observed in either abomasal or intestinal tissue sections ($P>0.05$ for all).

The results of the enumeration of mucin-producing cells stained with PAS and Ab-PAS are shown in Table 4.6. For PAS cells in abomasal tissue sections, there was an interaction between parasite treatment and lectin administration ($P=0.034$) reflecting a decrease in cell numbers in animals receiving either a parasite infection alone (+P-L compared to -P-L) or lectin administration alone (-P+L compared to -P-L) that was not observed in animals receiving both parasite infection and lectin administration (+P+L). In

addition there tended to be an interaction between parasite infection and lectin administration in Ab-PAS cells ($P=0.064$) which reflected a decrease in Ab-PAS cells in animals receiving lectin alone (-P+L) but an increase in cell concentration in animals concurrently receiving parasite infection and lectin administration (+P+L). No significant effects of infection, lectin administration or interactions were observed for either PAS or Ab-PAS cells in the small intestine

Table 4.4. Arithmetic mean \pm s.e. concentrations (cells per mm²) for eosinophils in abomasal and small intestinal tissues retrieved from lambs not parasitised (-P) or after 42 days of infection with *T. circumcincta* and *T. colubriformis* (+P), with (+L) or without (-L) lectin treatment and, stained using carbol-chromotrope.

Treatments	Eosinophils (cells per mm ²)	
	Abomasum	Small intestine
-P-L	0.2 \pm 0.1	13.2 \pm 4.1
-P+L	0.1 \pm 0.09	28.6 \pm 3.4
+P-L	9.9 \pm 3.8	23.1 \pm 4.4
+P+L	32.8 \pm 4.2	51.9 \pm 3.8
Effects		
Lectins	$P=0.001$	$P=0.001$
Infection	$P=0.001$	$P=0.001$
Lectin x Infection	$P<0.001$	$P=0.087$

Table 4.5. Arithmetic mean \pm s.e. concentrations (cells per mm²) for mucosal mast cells (MMC) in abomasal and small intestinal tissues retrieved from lambs not parasitised (-P) or after 42 days of infection with *T. circumcincta* and *T. colubriformis* (+P), with (+L) or without (-L) lectin treatment, stained using toluidine blue.

Treatments	Mucosal mast cells (cells per mm ²)	
	Abomasum	Small intestine
-P-L	189.2 \pm 8.7	216.6 \pm 9.7
-P+L	221.4 \pm 9.5	252.8 \pm 9.1
+P-L	224.5 \pm 10.1	224.2 \pm 8.7
+P+L	166.4 \pm 10.3	186.5 \pm 8.2
Effects		
Lectins	<i>P</i> =0.591	<i>P</i> =0.975
Infection	<i>P</i> =0.681	<i>P</i> =0.209
Lectin x Infection	<i>P</i> =0.071	<i>P</i> =0.117

Table 4.6. Arithmetic mean \pm s.e. concentrations (cells per mm²) of PAS staining cells and Ab-PAS staining cells in abomasal and small intestinal tissues retrieved from lambs not parasitised (-P) or after 42 days of infection with *T. circumcincta* and *T. colubriformis* (+P), with (+L) or without (-L) lectin treatment.

Treatments	PAS staining cells		Ab-PAS staining cells	
	Abomasum	Small intestine	Abomasum	Small intestine
-P-L	80.3 \pm 5.2	27.8 \pm 1.8	106.1 \pm 5.9	37.3 \pm 1.9
-P+L	68.0 \pm 5.3	31.0 \pm 1.9	82.4 \pm 5.6	36.2 \pm 2.0
+P-L	65.5 \pm 5.4	30.0 \pm 1.9	98.4 \pm 6.6	28.1 \pm 1.6
+P+L	98.7 \pm 6.4	27.2 \pm 1.5	124.4 \pm 7.8	44.5 \pm 2.9
Effects				
Lectins	<i>P</i> =0.311	<i>P</i> =0.958	<i>P</i> =0.929	<i>P</i> =0.138
Infection	<i>P</i> =0.439	<i>P</i> =0.833	<i>P</i> =0.192	<i>P</i> =0.926
Lectin x Infection	<i>P</i> =0.034	<i>P</i> =0.451	<i>P</i> =0.064	<i>P</i> =0.093

Immunohistochemical analysis of the T cell populations in abomasal and small intestine tissue sections are given in Table 4.7. Overall, Pan T cells were increased due to parasite infection in abomasal tissue sections ($P=0.013$) but not small intestine tissue sections ($P=0.662$) and were not influenced by lectin administration ($P>0.05$ for both). For T helper cell populations, concentrations in abomasal tissue sections were influenced by both parasite infection ($P<0.01$) and lectin administration ($P=0.051$). In addition, there was a tendency for an infection x lectin administration interaction which reflected a lesser increase due to parasite infection in animals concurrently receiving parasites and lectin (+P+L) compared to animals receiving parasite infection alone (+P-L) ($P=0.06$). There were no significant effects of treatment observed for either Pan T cells or T helper cells in small intestine tissue section ($P>0.05$ for all). Figure 4.3 (A-F) show photographs of tissue sections demonstrating the significant changes due to the interaction lectin x parasite infection in the population of eosinophils, PAS-positive cells (mucin-producing cells) and T helper cells from the abomasum.

Table 4.7. Arithmetic mean \pm s.e concentrations (cells per mm²) of Pan T cells (CD3) and T helper cells (CD4) in abomasal and small intestinal tissues retrieved from lambs not parasitised (-P) or after 42 days of infection with *T. circumcincta* and *T. colubriformis* (+P), with (+L) or without (-L) lectin treatment.

Factors	Pan T cells (cells per mm ²)		T helper cells (cells per mm ²)	
	Abomasum	Small intestine	Abomasum	Small intestine
-P-L	46.2 \pm 3.7	210.5 \pm 8.8	9.9 \pm 0.7	94.3 \pm 10.4
-P+L	49.7 \pm 6.0	173.0 \pm 11.1	9.7 \pm 0.7	85.4 \pm 23.5
+P-L	70.1 \pm 4.2	172.9 \pm 9.4	29.3 \pm 10.4	81.0 \pm 10.2
+P+L	76.0 \pm 4.3	194.8 \pm 10.2	18.3 \pm 1.8	77.6 \pm 10.8
Effects				
Lectins	<i>P</i> =0.614	<i>P</i> =0.667	<i>P</i> =0.051	<i>P</i> =0.541
Infection	<i>P</i> =0.013	<i>P</i> =0.662	<i>P</i> <.001	<i>P</i> =0.298
Lectin x Infection	<i>P</i> =0.897	<i>P</i> =0.113	<i>P</i> =0.060	<i>P</i> =0.784

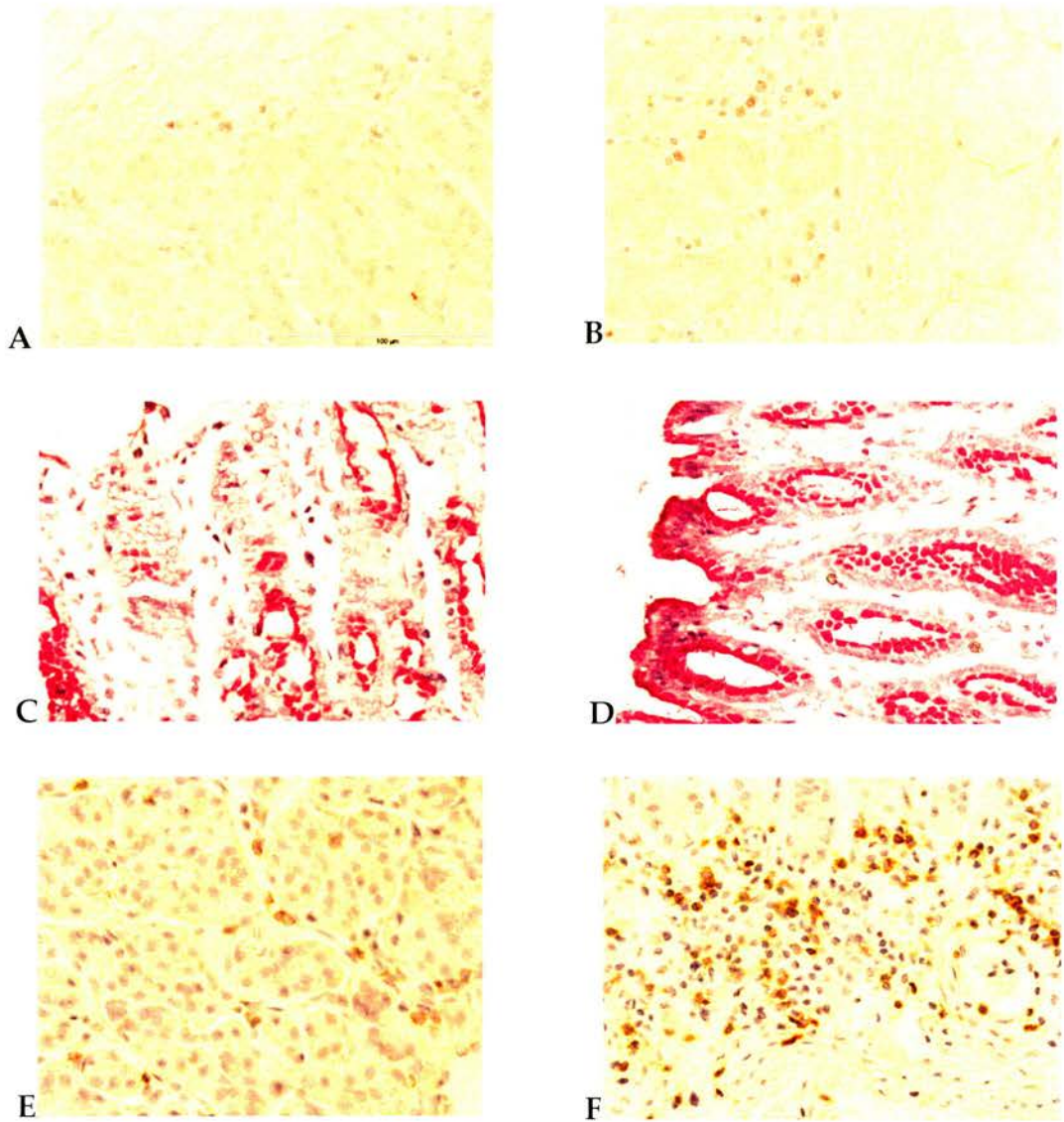


Figure 4.4. A-F. Photographs (40x) of tissue sections showing eosinophils (A, B), PAS-positive cells (mucin-producing cells) (C, D) and T helper cells (E, F), in sections retrieved from abomasa of parasitised animals with no lectin (+P-L) (A, C, E), compared to parasitised animals that received lectin (+P+L) (B, D, F). Significantly higher numbers of cells were observed due to the interaction lectin x infection.

4.3.8. Plasma analysis

Plasma total protein, albumin and globulin mean concentrations at day 0 were 63.7 ± 1.2 , 38.6 ± 0.7 and 25.1 ± 0.9 g/l, respectively. For all groups of lambs, there was an effect of time ($P < 0.001$) that reflected a temporary decrease of each blood parameter (-6-9%) only on day 7. There was no overall effect of either infection ($P = 0.223$) or lectin treatment ($P = 0.751$) on any plasma protein measurements. Mean plasma phosphorous concentration for all animals at day 0 was 11.0 ± 0.5 g/l and was not influenced by time, infection or lectin treatment ($P > 0.05$ for all).

4.4. DISCUSSION

The main results of this *in vivo* study are:

1. Lectins reduced the output of nematode eggs.
2. There was a tendency of the lectin to reduce the association of the larvae to the abomasal tissue, determined through the IVDC assay.
3. While there were no significant changes in cell populations from intestine, in the abomasum the interaction lectin x infection induced a significant increase in the number of eosinophils ($P < 0.001$), PAS-positive (mucin-producing cells) ($P = 0.034$) and had a tendency to increase the number of T helper cells ($P = 0.060$). No changes in mucosal mast cells numbers were observed in any of the animals.
4. Treatment with lectin did not induce any adverse clinical effects and there were no changes in serum protein or phosphate indicating possible toxic effects at this dose.

The study was primarily designed to investigate 'direct' anthelmintic effects upon a multi species worm population, using faecal egg counts and worm burden as the key parasitological comparators. The study covered a relatively short period of first exposure to infection (6 weeks) in order to minimize the confounding effects that the acquisition of immunity can have upon egg count and worm burden. Previous studies have shown that the various effector mechanisms that regulate parasite fecundity, larval establishment and adult persistence (Barger, 1987) do not begin to operate effectively during the initial phases of infection. Studies at Moredun Research Institute using the same breed of lambs and parasite isolates have shown that this is also the case for *T. circumcincta* (Seaton *et al.*, 1989b), *T.*

colubriformis (Sykes and Coop, 1976) and *Trichostrongylus vitrinus* (Seaton *et al.*, 1989a). Since the worm burdens of the control and lectin treated animals in this study were not significantly different for either genera it seems reasonable to assume that effective immunoregulatory mechanisms were not operational within the time-frame of the experiment. Under this assumption, it seems reasonable to conclude that the significant reductions in faecal egg count observed from day 25 can be attributed to a direct effect of the lectin on the parasitic population. However, such effects on fecundity were apparently transient in nature since they had disappeared by the time the animals were sacrificed and moreover there was no evidence of differences in *per capita* fecundity at post mortem. Although it is important to clarify that interpretations on fecundity must be cautious based on the fact of the mixed infection used in this study.

There was no evidence of any effective immunoregulation of the infrapopulation in this study, which is not surprising given the known complexity of these mechanisms (Jackson and Miller, 2006). However, allowing for the acknowledged immunostimulatory properties of lectins (Karmanńska and Michalska, 1978; Karmanńska *et al.*, 1996; Piekarska *et al.*, 2008) one cannot discount the possibility that there may have been some immunological changes induced by lectin treatment that might have subsequently influenced the rate of acquisition and expression of immunity.

The significant reduction in FEC, between days 25 and 36 was presumably mediated through a direct 'anti-worm' mechanism. The mechanisms underpinning this direct anthelmintic activity are not clear but might possibly be due to changes in behaviour induced by lectin binding that affected feeding, chemosensory or egg laying activity. Whilst it seems

reasonable to assume that the concentrations of PHA in the abomasum and intestine were sufficiently high to produce the apparent reductions in fecundity, the actual concentrations achieved in the gastrointestinal tract, and the proportion of lectin, which remains freely available to interact with the larvae, remains unknown. For example, it may be that some of the lectin is rapidly bound to polysaccharides in mucus lining the mucosal surfaces, or to carbohydrates on the luminal surface of epithelial cells. If this were the case, then one may expect a concentration gradient through the gastrointestinal tract, with the highest levels occurring in the proximal regions. Evidence that PHA binds to the mucosa has come from studies in rats where strong binding of PHA to the brush border in the small intestine and stomach has been shown (Pusztai *et al.*, 1990; Bardocz *et al.*, 1995; Linderoth *et al.*, 2006b) and in insects where PHA can bind to the gut (Habibi *et al.*, 1998, 2000). Treatment of rats with lectin has been shown to increase the number of mucin-containing cells in the intestine during repair after damage or trauma (Grant *et al.*, 2008). In the present sheep study, there were indications that mucus-containing cells in the abomasa were increased in infected PHA-treated (+P+L) animals, possibly indicative of an effect of PHA on the mucosa. Nevertheless, it is difficult to directly extrapolate these findings in rats to the present studies in sheep, with differences in species, parasite and dose of PHA. A further complicating factor is that, since the dosing was performed at a single time point/day, one would expect the concentrations to fluctuate, with the highest concentrations achieved within a few hours of lectin administration. If the latter was proven to be important, it should be possible to provide a more even or continuous dosage via the use of slow-release capsules or boluses, as has been demonstrated for anthelmintics (Bell and Thomas, 1992; Craig *et al.*, 2009). While the lectin may be affecting the L3 larval stage *in vivo*, the finding that there were no significant differences in

the worm burdens would suggest that there were no differences in larval establishment through the lectin treatment, as it was estimated. Given that there was no difference between the worm burdens of the control and lectin exposed lambs it also seems that there was no effect of the lectin treatment on pre-adult (L4) or adult (L5) worms either. Studies using a higher dose range of lectin might provide an answer as to whether lectins can directly affect worm establishment, growth and persistence and confirm which stages and key biological processes are most susceptible to disruption

A further important finding was that the dose of PHA employed (80 mg PHA/animal/d; 2.3 mg PHA/kg BW/d) in this study appeared to have no adverse effects, and there were no effects on the performance of the animals expressed as liveweight gain, blood measurements of serum protein and phosphate, or any histological evidence for damage of the abomasal or intestinal tissues. This dose rate was chosen because of concerns about the toxicity of PHA. The tolerance of sheep for PHA is unknown. Studies in rats have indicated that concentrations less than 250 mg/kg BW in this species are not harmful (Grant, 1999). However, in humans the lectin dose that can be tolerated without adverse reactions is ~2-3mg/kg BW/d (Hunter *et al.*, 2008). In rats, doses of PHA around 500mg/kg BW/d or higher impair growth and caused loss of body protein and body lipid, damage of the gut wall and overgrowth of *E. coli* (Pusztai *et al.*, 1991, 1993; Bardocz *et al.*, 1995; Grant, 1999). On the other hand, lower doses (≤ 200 mg/kg BW/d) of PHA acted as a potent growth factor for the small intestine, binding strongly to the brush border without triggering adverse or toxic responses in the animals (Bardocz *et al.*, 1996). These studies clearly demonstrate a dose response effect with PHA intake, with the highest concentrations studied being toxic. When

compared to these rat studies though, the doses that were used in the present lamb study are low but equivalent to those tolerated by humans.

In retrospect, higher doses of PHA could have been safely applied to lambs, with potentially more impact on the nematode infection. There is some evidence of a high tolerance of PHA in sheep or a number of studies have reported that adult sheep were able to consume considerable amounts of raw kidney bean without adverse reactions (Osborn *et al.*, 1985; Paduano *et al.*, 1995). In most cases, the lectin content of the seeds was not reported. However, Paduano *et al.* (1995) fed a named cultivar of kidney bean (>400g/d) to sheep with only limited effects on weight gain. The cultivar used is known to be of high lectin content [$\sim 20\text{g/kg DW}$] (Osborn *et al.*, 1985). In that study the sheep would have consumed approximately 8g of lectin/d [$>260\text{ mg/kg BW/d}$]. This suggests that sheep may have quite a high tolerance for kidney bean lectin. With this evidence and based on the results of our pilot study, it could be suggested that future studies should investigate the dose response of PHA in sheep and the pathological changes induced in response to these doses.

Even with the low dose of PHA given to the animals of this experiment, an important finding is the one obtained with the IVDC assay. This assay was developed to investigate the phenomenon of 'immune exclusion' for *T. circumcincta*, where incoming nematode larvae are prevented from migrating through the mucus layer and entering the crypt regions of the immune abomasal epithelium (Miller, 1984). Despite the extensive studies and wealth of information on the interaction between the host and this parasite, the precise mechanism for the full expression of this 'rapid immune exclusion' is unclear, but involves a range of acquired immune responses such as

antibodies, as well as innate factors (Meeusen *et al.*, 2005). Since the reaction is immediate, it indicates that the active factors are 'pre-formed' and maintained by continual stimulation with the worms. Studies with *H. contortus* have shown that cessation of dosing for 3 months resulted in the loss of this rapid immune response (Huntley *et al.*, 1992). The organ explant thus provides a functional *ex-vivo* model to study the phenomenon of rapid immune exclusion. The finding that PHA resulted in a tendency to increase the larval exclusion, suggests that there are aspects of the effector mechanism that are being altered and enhanced through this lectin treatment. These changes could relate to an increase in local antibody in the mucus (McClure, 2009), or the presence of anti-worm factors such as intelectin in mucus (French *et al.*, 2008).

Changes in the quality and quantity of mucus produced by goblet cells and also changes in MMC were not observed in this study. This was probably due to the low dose of lectin given to the animals, in addition to the short term of the experiment, which prevented us from finding a more complete immune response of the animals. However, changes in eosinophils in the abomasum and intestine and other cell populations from the abomasum of parasitised animals, in particular the increase in mucin-producing cells in infected/PHA-treated animals, could be interpreted as initial changes happening in the process of enhancement of the local immune response of animals receiving lectin.

Immunity against GIN develops over several weeks and months in sheep (Miller, 1984). It is therefore likely that the cellular changes important for the full expression of immunity had not been fully orchestrated at the time of termination of the present study. Perhaps more profound differences would

have been observed if the experiment had been continued for a longer period of time, say up to 12 weeks, although distinction between the effects of the hosts' immune response and the immunostimulatory properties of the lectin may make interpretation difficult. There is ample evidence for the critical involvement of both these cell populations in immunity and rapid expulsion of GIN in rats and ruminants (Rothwell, 1989) and any down regulation of these cells may have compromised the developing immune response. T helper cells include two major groups of cells according to the pattern of lymphokine secretion, Th-1 that secrete IL-2, interferon- γ and tumour necrosis factor, and Th-2 that secrete interleukins 4, 5, 6, and 10 (Kilpatrick, 1999). Lavelle *et al.* (2000) showed that some plant lectins dosed orally to mice are able to stimulate systemic and mucosal antibody responses and are strong mucosal immunogens. Although the present study was designed for 6 weeks duration, some major changes in the cell populations of eosinophils and PAS-positive cells were demonstrated in the abomasa. Both eosinophils and goblet cell hyperplasia are characteristic features of GIN infections, and the finding that PHA can enhance their numbers at the early stages of the immune response to GIN is an intriguing finding. Eosinophils are known effector cells related to the killing of infective larvae stages of GIN but have no effect on adult stages, both *in vitro* and *in vivo* (Meeusen and Balic, 2000). In *H. contortus* and in *T. circumcincta* infections, eosinophil infiltrations have been found to be closely associated with the tissue larvae *in vivo* (Balic *et al.*, 2002, 2003), further suggesting a possible direct anti-parasite effector function on larvae. Thus, increased populations of eosinophils and mucin-producing cells at the site of infection through treatment may represent an enhanced 'early stage' in the development of the immune response to *T. circumcincta*. However, since the cell counts in the present study come from a single time

point at slaughter, further kinetic studies are required to fully evaluate changes in these cell populations.

GENERAL DISCUSSION

This study has shown for the first time that lectins can have a significant impact, both directly and indirectly, on gastro-intestinal nematodes of sheep. The use of lectins to influence nematode infections in ruminants is an entirely novel finding that provides first proof of concept of their potential for use in this area.

To summarise the main findings of this study, *in vitro* studies were used to identify direct anthelmintic effects on the larval stages of nematodes and led to the identification of PHA-E3L as the most suitable candidate for use in an *in vivo* trial. The PHA-E3L lectin trial demonstrated direct effects upon FEC and also that there was a reduced ability of exsheathed *Teladorsagia* to become closely associated with abomasal tissues from lambs exposed to lectins. There was also evidence that PHA-E3L increased the numbers of eosinophils and PAS-positive cells in the abomasum, in response to parasite infection. The *in vivo* experiment using sainfoin hay also produced evidence of decreased FEC and increased intestinal cell populations of mucosal mast cells, eosinophils and Pan T cells in the sainfoin hay group compared to the grass hay fed lambs. These results all suggest that the intake of certain PSM's may influence either directly, or indirectly, the course of GIN infection.

Whilst the use of PSM for parasite control is an attractive prospect, particular for tropical and subtropical regions where there is an ample supply of PSM

rich plants, there are a number of important factors that also need consideration. There are three obvious routes for the application of nutraceuticals, firstly to simply to feed whole plants or their specific parts that are rich in PSM. Secondly one can semi-purify the active components and administer these directly or as a feed additive. The third and most complex route is to characterise the active components, to identify and purify them for use as 'conventional' anthelmintics or growth or immune enhancers. The potential of each of these options can be considered on the basis of their relative cost, efficacy, safety and applicability in any given environment. One of the major problems facing researchers in this area is the variability inherent in these plant products and their complexity that affects active concentration, bioavailability and efficacy (Figure 5.1.).

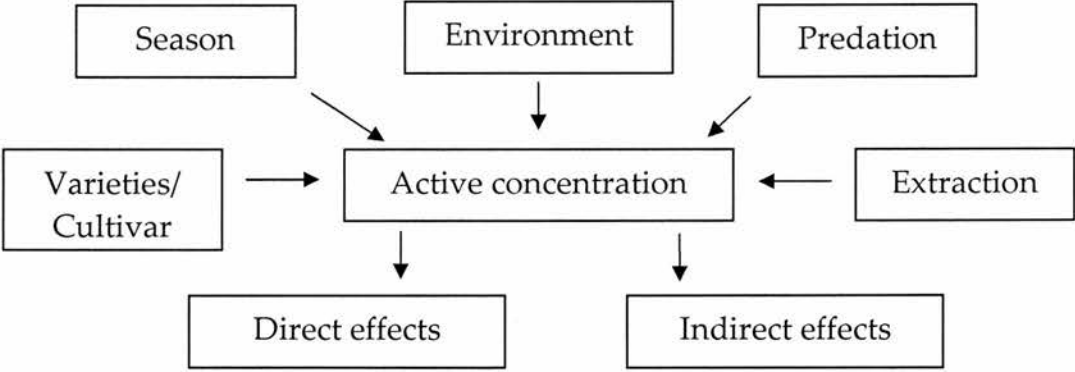


Figure 5.1. Some of the key factors affecting the concentration of bioactive compounds in plant secondary metabolites-rich plants and plant extracts that might influence direct anthelmintic activity.

Whilst the first approach has the value of simplicity, a lack of understanding regarding the active components and their concentrations imposes severe

limitations (Waller, 1997a; Hoste *et al.*, 2008b; Waghorn, 2008). Nevertheless for resource poor farmers this approach, using locally available forages and agricultural PSM rich by-products, has considerable merit. The second option of using semi-purified products may impose additional costs but offers the advantages of having a better defined product without the presence of other, possibly toxic, contaminants. Although fully characterising these plant products is expensive it has the advantages of providing uniformity of activity and an insight into the mode of action of the active factor. Moreover an understanding of the mode of action may not only provide novel anthelmintics and targets for anthelmintic therapy but may also lead to the identification of immuno-stimulatory properties which may have considerable benefits with regard to a range of non-parasitic diseases and disorders. For example it is known that, by increasing the turnover of epithelial cells, lectins protect against the damage induced by anti cancer chemotherapy in humans (Hunter *et al.*, 2008). This highlights the potential of these bioactive plant products to influence a whole range of biological processes outside of those that can directly or indirectly influence internal parasite populations.

5.1. Direct Mechanisms

Although the studies provide both *in vitro* and *in vivo* evidence of direct anthelmintic effects attributable to products derived from tannin rich plants such as sainfoin, *G. sepium*, *L. leucocephala* and lectins, the mechanisms underpinning these effects are not clear. Although there is evidence that both CT and lectins can bind strongly to nematode cuticles, the results from these studies suggests that whilst these two PSMs may have some shared effector

pathways, they may also have other unique modes of action. *In vitro* and *in vivo* studies in goats (Paolini *et al.*, 2003c; Bahuaud *et al.*, 2006; Brunet *et al.*, 2007) have shown that CTs have the capacity to affect the incoming larval stages and, by slowing the processes of exsheathment and establishment at the predilection site, can disrupt the vital initial stages of infection. However, since the *in vivo* experiment (Chapter 4) produced no evidence of any marked effects of PHA lectin on overall establishment it seems reasonable to suggest that, at the dose rate used in the study, lectins had no deleterious effect on those aspects of larval biology and behaviour that are known to influence establishment. Both lectins and CT are known to be able to affect the feeding behaviour of the pre-parasitic larval stages but it is not clear whether these effects also occur in the parasitic stages. Severe effects on feeding behaviour are also likely to have some consequence for the capacity of female worms to produce large numbers of eggs. However, if this were the case in the PHA study then one would have expected that suppression of egg laying would have persisted at least throughout the period of lectin administration and moreover that it might also be evident in the *per capita* fecundity calculations. Since this was not the case then consideration needs to be given to the possibility that worms can develop some tolerance or resilience against the effects on reproductive behaviour that are either directly or indirectly mediated. If this is indeed the case then this might have important implications for the use of bioactive plant products to control these two economically important species of nematode. However, without a clear understanding of the mode(s) of action of PSM, the identity of the key active components and tools to measure parasite susceptibility to them, it is clearly very difficult to design studies to investigate changes in susceptibility that occur over time.

5.2. Indirect mechanisms

Despite the fact that both *in vivo* studies in this thesis were relatively short term experiments with low potential to investigate effects of PSM on the development of immunity, both studies provided clear evidence of enhancement of cell populations involved in immunoregulation. In addition to the direct effects of lectin described above, this study has made the intriguing finding that the oral dosing of PHA induced changes in the eosinophils and PAS-positive cells in the abomasum indicating the potential for indirect mechanisms of control via the enhancement of the natural immune mechanism(s). Eosinophils have been considered to be effector cells involved in the rejection of the larval stages of GIN, although the precise mechanism of this effect is unknown and their overall importance in immunity remains controversial (Balic *et al.*, 2002, 2003). Enhanced activity of mucin-producing cells have been also been found to be related with parasite immunity, since mucins and mucin-like molecules are involved in helminth-host interactions (Theodoropoulos *et al.*, 2001), possibly through the entrapment of larvae which aids their expulsion (Miller *et al.*, 1985).

Given that these changes through lectin treatment are beneficial in terms of immunity, then one can postulate various situations where boosting the immune response to GIN in sheep is important. For example, as has been discussed before, it is now well established that young lambs are relatively slow to develop an effective immune response which controls GIN worm burdens, when compared to adult (>1 year old) animals (Jackson *et al.*, 2004; Torres-Acosta and Hoste, 2008). An enhancement in the development of this protective mechanism would bring benefits to the health of the lambs at a

stage of development when they are most vulnerable, reducing FEC earlier and reducing pasture contamination. Another important application would be to overcome the peri-parturient breakdown in immunity to nematode parasites in sheep. In this phenomenon, ewes which have developed immunity to GIN proceed to shed higher numbers of eggs in the faeces during or just after the birth of the lambs and this can add significantly to the pasture contamination (Coop *et al.*, 1990). The underlying mechanisms of this peri-parturient rise remain unclear, and may be a result of the loss of immunity through lack of worm challenge in ewes, or a breakdown in the underlying mechanisms of immunity (Sykes *et al.*, 2007). This breakdown can be reversed, or ameliorated, with the addition of protein to the ewes, suggesting that there is a hierarchal order of priorities for nutrients and that growth of the lamb has priority over the immune responses, resulting in a diversion of essential nutrients for growth (Sykes *et al.*, 2007). Clearly, if the addition of lectin can boost the immune response during this peri-parturient period, then this would reduce this pasture contamination.

One further example is the possible use of lectins as a targeted approach to boost immunity in lambs that are generally slow to develop immunity and have higher FEC, compared to others within the same flock. This approach is being considered for anthelmintics, based on the performance (measured as efficiency of production based around liveweight gain), but within a flock there are animals that develop immunity at different rates and consequently a wide range of FEC and consequent economic performance. Boosting the immunity through the selective and judicious use of lectins may provide a more cost effective approach for their use.

While knowledge on the precise effects of lectin treatment, the target cell population(s) and the mechanism of how this occurs could be of importance in controlling gastrointestinal parasites of sheep, it should be recognised that boosting immunity in broad terms may be relevant for other parasites, diseases and conditions for animals, as well as for man. The interest in 'probiotics', particularly for human health, is now intense with various claims for benefits due to the intake of various food additives such as vitamins and benign bacteria. This interest highlights the potential for this area of research, and it is conceivable that lectins could become an important 'pro-biotic' in their own right. From studies already conducted (Grant, 1999), it is apparent that lectins can have a direct effect on cells or organs other than the immune response, since the oral intake in humans and rats has shown specific increases in mucosal epithelial turnover which protects from damage due to chemotherapy. Thus, the benefits of lectins may be broadened to include other target cells or organs, where their use could control the damage due to diseases and disorders which at present we are unable to prevent or cure. For example, in addition to cancers, diseases such as rheumatoid arthritis, psoriasis and allergies are complex conditions where modern medicine has failed to provide reliable cures, and the selective treatment of defined lectins may beneficially influence the course of these diseases.

Of course, the above provides a speculative positive assessment for the use of lectins, but their potency comes at a cost since many are toxic. Indeed PHA has been shown to be toxic to rats and humans at concentrations of >500 or 2mgs/Kg BW, respectively, and PHA efficacy is highly dose dependent (Pusztai, 1991; Pusztai *et al.*, 1993; Grant, 1999). Some lectins are known to be highly toxic, such as ricin, which binds to receptors on eukaryotic cells and is consequently designated as a biological warfare agent (Uzawa, 2009). This

emphasises the importance of performing careful *in vitro* studies with unknown lectins, before embarking on *in vivo* animal studies. However, it is relevant that the toxic ricin component could be reduced in castor seed cake, a by-product of the vegetable-oil industry, to provide a suitable substitute for conventional soybean meal as a protein rich feed for sheep (Bainter *et al.*, 1993). Thus, even complex plant extracts containing highly toxic components, may be able to be utilised in a productive manner and indicates that their toxicity *per se* is not necessarily a barrier to their effective use.

5.3. Future research

From the above it is apparent that further research is needed to confirm the range of activity and potential for this fascinating group of carbohydrate binding proteins. Since almost all plants appear to have lectins in greater or lesser amounts, the range and diversity of these and their interactions is almost limitless. The present study has focussed on just a few, well-defined, lectins but has demonstrated the potential for their novel use in controlling GIN in sheep. For PHA, dose response studies are needed to define the optimum dose, and duration of dosing. As has been mentioned, there is evidence that sheep are able to tolerate much higher levels of PHA than humans, which may benefit their use for GIN control. Fundamental questions remain, however, on their mode of action. Regarding the direct effects, immuno-electron microscopy localisation studies with labelled PHA or other lectins may provide some answers as to the target organs or cells on the nematode, and there may be stage-specific targets i.e. the reactive carbohydrates may be different, or have different locations, on larval compared to adult worms. Modern proteomic methods may assist in these

studies, for example it may be possible to identify the reactive glycoproteins using labelled lectins, through Maldi-tof analysis of the nematode molecules. On a practical level, it will also be important to determine the efficacy with different species of nematodes, as well as information on the dosage and frequency of delivery. For the latter, the possible use of slow release boluses may be considered, to provide a more uniform delivery and consistent dose of lectin at the site of importance. The survival of the lectin in an intact and functionally active form through the gastrointestinal tract is also relevant, and an experiment to investigate this aspect could be easily performed. Lability and storage of any biologically active agent is always important, particularly where the lectin has been purified since activity may be less robust than in a 'complex or semi-purified' form where associated proteins may protect from degradation. Equally important is the requirement for studies to define the activity of lectins on the host. For GIN in sheep, both the local (mucosal) and systemic responses or consequences of the oral dosing of lectins need to be investigated. The current work has necessarily focused on the mucosa within the gastrointestinal tract; since lectins may be absorbed intact in an active form it will be important to investigate local as well as systemic effects. This will include a careful analysis of organs throughout the body, and particularly those involved in the immune response such as the spleen and lymph nodes. Detailed cell and cytokine responses of these organs would provide a more holistic view of the effects of lectin- and comparative microarray studies could provide a means to study the effects of treatment on many genes at the same time. Other fundamental questions also require investigation, such as the possible variation in response of individual animals to the same dose, or to different lectins, and the responses to treatment in different breeds. The latter may be particularly important for breeds which have been bred and predominate in defined regions such as the

tropics, and which may have quite different responses to and/or efficacies arising from treatment with a given lectin than those from other, for example, temperate regions. In addition to the immune responses, important studies are also required on the effect of lectins on performance of animals, and again, this performance including weight gain may differ between individuals and breeds. At high concentrations, oral supplementation of lectins may suppress appetite, indeed an extreme example is where rats would not eat and starved after eating the mushroom *Pleurotus ostreatus* lectin, which was found to have a highly potent intake-suppressing activity which was lectin dose-dependent (Kawagishi *et al.*, 2000). This effect illustrates the potency that some lectins exert, and the caution that must be taken in such *in vivo* studies.

To summarise, the present studies have provided evidence for the first time that lectins may be powerful tools to influence the course of GIN infection in sheep. The effects and mechanisms of these potent molecules require much further investigation, but their potential to both directly and indirectly affect the parasites through enhanced immunity, suggests that this area is likely to be a fruitful area for research in the future.

5.4. Potential applications

Whilst there can be no doubt that many farmers recognise nematodoses as one of the major constraints limiting ruminant production and animal welfare, the measures that farmers are willing to adopt to combat them vary enormously between different farms, regions and countries. Crucial, often cost sensitive, issues such as practicality, applicability,

affordability and complexity, with regard to the extent of knowledge required to apply a strategy, influence farmers' decisions regarding how they approach worm control. Whilst there is now a considerable body of research that has focussed on the role that PSM and in particular CT can play in regulating gastrointestinal nematodes (Bahuaud *et al.*, 2006; Hoste *et al.*, 2006), it is evident that research on the potential role of lectins is in its infancy, particularly regarding their potential to enhance immunoresponsiveness. The studies in this thesis have shown that the PSMs in sainfoin and in semi-purified lectin products can have some effect upon the parasites and also induce changes in host immune cell populations. These characteristics, together with the nutritional benefits that some bioactive forages offer has led people to describe these plants as nutraceuticals (Waller, 1997a, 2006; Torres-Acosta and Hoste, 2008). The nutritional components of PSM rich plants maybe particularly important for resource poor farmers in tropical areas where the availability of both protein and energy can limit productivity (Krecek and Waller, 2006). Studies using simple urea/molasses blocks in Asia (Knox and Steel, 1996) and energy supplementation in areas of Mexico (Torres-Acosta *et al.*, 2006) have shown benefits with regard to performance and resistance and resilience against parasite infections.

There are a number of examples of how nutraceutical plants can be exploited as part of a parasite control strategy e.g. conserved sainfoin (Hoste *et al.*, 2005; Heckendorn *et al.*, 2006), *Sericea lespedeza* (Terril *et al.*, 2007) and chicory (Athanasiadou *et al.*, 2007). One of the major problems confronting this approach is that the effects appear to be parasite and host specific, for example Heckendorn *et al.* (2007) showed that in sheep conserved sainfoin products affected the abomasal species *H. contortus* but had only minor

effects on the intestinal species *C. curticei*. By way of contrast Paolini *et al.* (2005b) working with sainfoin in goats showed effects on the intestinal species *T. colubriformis* but not on the abomasal nematode *H. contortus*. These contrasting findings suggest that to successfully apply a nutraceutical approach requires some understanding of the epidemiology of the nematode species present on a farm. The need to acquire this knowledge is a barrier for many farmers since they have, since the 1960s (McKellar and Jackson, 2004), been able to rely upon the availability of highly effective broad spectrum anthelmintics. In recent years the widespread emergence of multiple anthelmintic resistance (Waller, 1994, 1997b; Jackson and Coop, 2000), the need to maintain parasite populations in refugia (unexposed to anthelmintics) (Jackson and Miller, 2006) and the move towards minimal chemical intervention and organic farming has shifted this balance and has led to a growing interest in alternative forms of control.

The well documented threshold dependent anti-nutritional effects of PSM (D'Mello, 1992; 2000), as already discussed, are also an issue since it affects the safe use of nutraceutical plants and their products. Since many of the PSM are defensins, their concentrations in plant tissues may vary widely in a single plant species in an area (Hoste *et al.*, 2008b) and within and between seasons. Obviously the use of conserved or purified products with defined levels of activity below those associated with toxicity can overcome this problem, but since this has cost implications it may be of little value for small holders with limited resources. Despite these obvious challenges there can be no doubt that bioactive forages will play an increasing role in future as one component in integrated pest management systems.

One of the most intriguing findings seen in the two animal studies was the evidence of the early alteration in immune responses in lambs receiving sainfoin or PHA lectin. The short duration of both these studies meant that animals were killed at a time when one would not expect effective immunoregulatory mechanisms to have become established. Whilst the effects on local cellular response components seen in the sainfoin study might have either a nutritional and/or an immunostimulatory basis, those seen in the lectin study may well have had an immunostimulatory rather than nutritional basis. Enhancing the host immune response through genetic selection, optimised nutrition or vaccination has for many years been recognised as an important means of controlling nematode infections and reducing our reliance upon anthelmintics. Making the assumption that the changes induced by lectins enhance parasite immunoregulation then these 'immunoceuticals' might play a valuable role in worm control. Conceivably the major role for immunoceuticals would be to act to provide a more rapid acquisition of immunity in young stock or to promote the maintenance of immunity in older stock either during the peri-parturient period or at times of nutritional stress, when, as discussed above, acquired immunity wanes. The issue of the cost to the host of acquiring immunity is an interesting one, since studies by Greer *et al.* (2008) have shown that many of the loss in fattening lambs attributable to infection with *Teladorsagia* and *Trichostrongylus* appear to be a consequence of the host immune response. If research can identify plant derived immunoceuticals that can be used to either turn these complex mechanisms on or off then it is even conceivable that in future this form of immunomanipulation might play an important role in maximising livestock production. For example it might be desirable to maximise the rate of acquisition of immunity in replacement and breeding

stock but the reverse might apply to fattening lambs where there could be some production benefit in minimising host immune responses.

However, the reality is that these exciting prospects linked to manipulating host immunity remain long term goals and that they will only be realised if research in this area continues to receive both the attention of the research community and more importantly adequate funding to underpin it.

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PUBLISHED WORK

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